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## **Idiopathic erythrocytosis is associated with a risk of thrombosis - a retrospective case series review**

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1

**Karyotype is an independent prognostic factor in adult acute lymphoblastic leukaemia**

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Pre-treatment cytogenetics is a known predictor of outcome in haematological malignancies. However, its usefulness in adult acute lymphoblastic leukaemia is generally limited to the presence of the Philadelphia chromosome (Ph) because of the low incidence of other recurrent abnormalities and the rarity of the disease in this age group. We present centrally reviewed cytogenetic data from adult patients enrolled on the Medical Research Council UKALLXII/Eastern Cooperative Oncology Group 2993 Trial. Ph-positive patients ( $n = 267$ ) had a significantly inferior 5-year overall survival (OS) compared with Ph-negative patients ( $n = 1106$ ): 22% (95% CI 17–27%) vs 41% (38–44%) [ $P = 0.0001$  adjusting for age, gender and white cell count (WCC)]. Within the Ph-negative cohort, the following chromosomal abnormalities were associated with a poorer outcome:  $t(4;11)(q21;q23)$ ,  $n = 54$  (7%), OS 24% (13–36%),  $P < 0.001$ ;  $t(8;14)(q24.1;q32)$ ,  $n = 16$  (2%), OS 13% (2–33%),  $P < 0.001$ ; complex karyotype (five or more chromosomal abnormalities),  $n = 41$  (5%), OS 28% (15–43%),  $P = 0.027$ ; low hypodiploidy/near triploidy (Ho-Tr),  $n = 31$  (4%), OS 22% (9–38%),  $P = 0.001$ . In contrast, patients with high hyperdiploidy or  $del(gp)$  had a significantly improved outcome:  $n = 77$ , OS 53% (41–64%),  $P = 0.015$  and  $n = 71$ , OS 58% (46–69%),  $P = 0.032$  respectively. We used a Cox proportional hazards model to assess the prognostic relevance of cytogenetic variables within the Ph-negative cohort in the context of other established survival indicators: gender, age, WCC and T-cell status. The adverse effect of  $t(8;14)$ , Ho-Tr and complex karyotype was shown to be independent of these risk factors both within the overall cohort and a reduced cohort which excluded patients undergoing a bone marrow transplant. The observation that Ho-Tr and, for the first time, karyotype complexity confer an increased risk of treatment failure demonstrates that cytogenetic subgroups other than the Ph can and should be used to risk stratify adults with ALL in future trials.

2

**Deletion of 16q identified by FISH is an independent adverse prognostic marker in multiple myeloma**

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Deletion of 16q ( $del(16q)$ ) has been identified in 15% of newly diagnosed myeloma but the prognostic impact has not been determined. We performed FISH on CD138 selected plasma cells from 861 newly diagnosed patients with multiple myeloma from the LRF UK Myeloma Forum Cytogenetics Database.  $Del(16q)$  occurred in 168/861 cases (19.5%) and was significantly associated with deletion 13q (54.8% vs 43.5%,  $P = 0.009$ ), deletion of IgH (19.6% vs 8.7%,  $P < 0.001$ ), deletion 17p (20.7% vs 7.2%,  $P < 0.001$ ) and non-hyperdiploid status (47.9% vs 40.3%,  $P = 0.043$ ). Clinical and survival data was available in 505 patients. Median age was 65 years (range 33–92) and median follow-up was 19 months.  $Del(16q)$  showed no association with baseline clinical and demographic parameters but was associated with a significantly worse overall survival (median survival 36 months vs not reached,  $P = 0.025$ ). Moreover,  $del(16q)$  conferred additional adverse impact in combination with the known poor risk cytogenetic factors  $t(4;14)$  and deletion 17p ( $del(17p)$ ). Median survival for  $del(16q)$  and  $t(4;14)$  was 13 months,  $del(16q)$  alone 36 months,  $t(4;14)$  alone not reached,  $P = 0.001$ . Median survival for  $del(16q)$  and  $del(17p)$  was 17 months,  $del(16q)$  alone 36 months,  $del(17p)$  alone not reached,  $P = 0.003$ . Multivariate analysis confirmed that  $del(16q)$  retained independence as an adverse prognostic marker ( $P = 0.003$ ) along with  $t(4;14)$ ,  $t(14;16)$ , light chain isotype, WHO performance status, ISS and age.

Integration of gene mapping with global gene expression data in a subset of 55 cases identified two potential tumour suppressor genes located on 16q, CYLD and WWOX. We have shown that loss of CYLD dysregulates the NF $\kappa$ B pathway and loss of WWOX dysregulates apoptosis via p73, both of crucial importance in myeloma biology. WWOX is also a common fragile site gene and deletions at other common fragile sites were identified that may also contribute to myeloma pathogenesis. An update of this mapping and expression data including the NF $\kappa$ B signature associated with 16q deletion will be presented.

3

**GCS-100: a novel therapy for myeloma inhibits Mcl-1, bcl-xL and cell cycle proteins**

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Marrow stromal cells in myeloma secrete cytokines that stimulate anti-apoptosis, drug resistance, cell cycle and metabolic pathways. Disruption of these networks can lead to myeloma cell death making them attractive targets for novel therapies. The modified citrus

pectin, GCS-100 has been used safely in Phase I studies for solid tumors, induces cell death in lymphoma via blocking galectin-3/Bcl-2 dimerisation and in myeloma cells by mechanisms not fully understood. This study aimed at further elucidating the mechanism of action of this novel agent in myeloma. GCS-100 induced apoptosis in a dose and time dependent manner in myeloma cell lines RPMI8226, U266 and OPM2 with associated significant increase in Go/sub-G1 cells. Treatment led to a marked time and dose dependent reduction of the crucial anti-apoptotic proteins mcl-1 and bcl-xL. NFkappaB activation in myeloma cells is associated with proliferation and adhesion molecule upregulation. GCS-100 led to a time-dependent reduction in activated IkappaBalpha and p65NFkappaB. Furthermore pre-treatment of myeloma cells with GCS-100 inhibited IkappaBalpha activation by exogenous TNFalpha. Similarly, treatment with GCS-100 led to a reduction in the amount of activated Akt (a cell cycle regulator) and inhibited IGF-1 associated activation of Akt. The effect of GCS-100 on cell cycle regulatory proteins revealed downregulation of cyclin D1, p16<sup>INK4A</sup> and CDK6 at 24 hours, however, there was no change in expression of CDK4 and p15<sup>INK4B</sup>. The p21<sup>CIP1</sup> was upregulated with a corresponding decrease in protein levels of cyclin E2 and CDK2 but with no change in p27<sup>KIP1</sup>. Studies are currently ongoing for primary cells. In conclusion GCS-100 is a novel complex carbohydrate that reduces proliferation and induces apoptosis by down-regulating crucial anti-apoptotic proteins, cell cycle regulators and signalling proteins and may also act by interfering with the myeloma cell microenvironment. Phase I studies in myeloma have been commenced.

## 4

#### Restricted MHC phenotypes in splenic marginal zone lymphoma

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Immunoglobulin heavy chain variable gene sequencing in splenic marginal zone lymphoma (SMZL) reveals biased usage of the VH1-02 gene, present in 30% of cases, suggesting that stimulation by a specific antigen may have a role in the pathogenesis of this disease. SMZL is thought to arise from normal splenic marginal zone B cells which in health mediate rapid but short lived immunity to bacterial antigens. Although these responses are generally thought to be T-independent both normal and malignant splenic marginal zone B cells may undergo somatic hypermutation (SHM). To investigate whether SHM in SMZL occurs through a T-dependent or T-independent pathway we looked for evidence of MHC restriction. Medium resolution HLA typing was performed by PCR-SSO and the phenotypes of 18 cases of SMZL utilising the VH1-02 gene and 18 non-VH1-02 SMZL's were compared with 1667 normal controls, 17 cases of CLL utilising VH1-02 and 40 VH3-21 CLL's by Fisher's exact test with Bonferroni's-correction. Sixty one percent of the VH1-02 SMZL's had an HLA-DR15 phenotype compared to only 26% of controls ( $P = 0.0022$ ). Both VH1-02 SMZL's that did not express HLA-DR15 and non-VH1-02 SMZL's were associated with HLA-DR4 ( $P = 0.0023$ ). In contrast there was no MHC restriction in cases of CLL utilising either the VH1-02 gene nor the VH3-21 gene. These findings support the hypothesis that SMZL derives from a marginal zone B cell that has responded to a T-dependent antigen.

## 5

#### The 13q and 11q B-cell chronic lymphocytic leukaemia deleted regions derive from a common ancestral region in the zebrafish on chromosome 9

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Loss of the long arm of chromosomes 11 and 13 are the commonest cytogenetic abnormalities for patients with B-cell chronic lymphocytic leukaemia (B-CLL) and suggest the presence of as yet unidentified tumour suppressor genes. The use of small vertebrate organisms, such as the zebrafish, as models of diseases associated with chromosomal deletions enables the functional analysis of potential causative genes. In this study, the evolutionary conservation between the zebrafish and human genome is investigated for the 13q14 and 11q22-23 regions deleted in B-CLL. Zebrafish orthologues have been identified and radiation hybrid mapping performed to confirm their chromosomal location and define regions of conserved synteny. The 13q14 region was syntenic with two main regions in the zebrafish genome, namely chromosomes 1 and 9. The majority of zebrafish orthologues to 11q22-23 were found on chromosomes 5, 15 and 21. One region within an area of 22.02 cR on zebrafish chromosome 9 (approximately 3260 kb) is of potential interest. Within chromosome 9, five genes and two microRNAs were identified with shared synteny to the smallest 11q22-23 and 13q14 critically deleted regions (two genes to human chromosome 11, three to human chromosome 13 and two chromosome 13 microRNAs). The critical region on zebrafish chromosome 9 maps to the minimal deleted region for both human chromosomes, suggesting a common ancestry for the B-CLL tumour suppressor genes. This is further supported by analysis of the chicken genome where the same 5 genes from 13q14 and 11q22-23 (C13orf1, RFP2, FLJ11712, FDX1, ARHGAP20) lie within a 10.04 Mb region on chromosome 1. Target-selected mutagenesis for knock-outs of genes in this region of zebrafish will allow analysis of their *in vivo* potential for lymphoproliferation. Our study provides an explanation for involvement of both 11q and 13q in B-CLL and potential to develop animal models for this common lymphoproliferative disorder.

## 6

#### Five CCAAT-enhancer-binding-protein gene family members are deregulated by the immunoglobulin heavy chain locus in B-cell precursor acute lymphoblastic leukaemia

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Chromosomal translocations lead to oncogene activation in a significant number of haematological malignancies. Those involving the immunoglobulin heavy chain locus, *IGH*, at chromosome band 14q32 are frequently observed in B-cell malignant proliferation. A small number have been described in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL). However, their biological and clinical significance is currently unknown. Detailed fluorescence *in situ* hybridisation (FISH) and molecular studies were carried out on a series of BCP-ALL patients with chromosomal abnormalities involving 14q32. Novel and recurrent translocations affecting

different chromosomes were highlighted. Refined FISH mapping identified putative *IGH* partner genes at, or flanking, the translocation breakpoints. Four translocations: two previously reported,  $t(14;19)(q32;q13)$ ,  $t(8;14)(q11;q32)$ , and two novel,  $t(14;14)(q11;q32)/inv(14)(q11q32)$  and  $t(14;20)(q32;q13)$ , were identified. Molecular analyses showed that four different members of the *CAATT enhancer binding protein (CEBP)* gene family were involved: *CEBPA* (19q13,  $n = 9$ ), *CEBPD* (8q11,  $n = 8$ ), *CEBPE* (14q11,  $n = 3$ ) and *CEBPB* (20q13,  $n = 2$ ). One patient with a  $t(14;19)(q32;q13)$  was observed to involve the fifth family member *CEBPG* (19q13,  $n = 1$ ). Breakpoints were located within the 3' untranslated region (UTR) of *CEBPA* and either 3' UTR or 5' of *CEBPD*, whereas breakpoints in 8q11 were ~30 kb centromeric of *CEBPD*. Where material was available, over-expression of target genes was shown by quantitative real-time PCR. Overall, this study has demonstrated for the first time the involvement of five members of the same gene family in a single subtype of haematological disease. It has indicated that transcriptional upregulation of *CEBP* gene family members, by juxtaposition to *IGH*, is important in BCP-ALL: a mechanism in complete contrast to that involving *CEBPA* in acute myeloid leukaemia.

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### Non-hodgkin's lymphoma (NHL) cells induce a local and systemic regulatory T-cell response

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T regulatory (Treg) cells, especially CD25+, are a type of lymphocyte that suppress immune responses and may be important in mediating escape of malignant cells from immune surveillance. In order to investigate their importance in NHL, we enumerated Treg cells in peripheral blood (PBMC) and involved tissues from 30 patients at diagnosis. CD25+ FoxP3+ CD127<sup>low</sup> CD4+ T reg cells were increased significantly in PBMC (patients' median = 20.3% of CD4T cells [ $n = 20$ ] vs healthy control median = 3.16% of CD4T cells [ $n = 13$ ];  $P < 0.001$ , rank sum test). This proportion is higher than recorded in any other malignancy and may explain the global immunosuppression seen in NHL. Furthermore, Treg cells from PBMC significantly correlated with serum lactate dehydrogenase, a tumour bulk marker ( $r^2 = 0.84$ ,  $P < 0.001$ ). Poor T-cell responses to control stimuli by PBMC from patients with NHL were reversed by depleting CD25+ cells with magnetic beads or by addition of anti-CTLA-4. As a high percentage of Treg cells were also present in involved tissues (patients' median = 38.8% of CD4T cells [ $n = 15$ ] vs reactive nodes' median = 11.6% of CD4T cells [ $n = 2$ ];  $P = 0.02$ , rank sum test), we determined if tumour cells could induce a Treg phenotype. We incubated the CD25- PBMC fraction with tumour cells *in vitro* for five days. A dose and time-dependent Treg phenotype (CD25+ FoxP3+) induction was seen (number = 6, maximum induction of 86.76%). Less effective induction was seen when these populations were separated in transwells. These 'induced Treg cells' were FACS sorted and suppressed effector T-cell proliferation. We conclude that NHL cells are powerful inducers of

Treg cells. These suppressive cells circulate and may induce active immune tolerance both systemically and within the tumour microenvironment, thus representing a new therapeutic target in NHL.

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### Report of the UKCLL02 trial: a phase II study of subcutaneous alemtuzumab and fludarabine in patients with fludarabine refractory CLL (on behalf of the NCRI CLL trials sub-group)

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Fludarabine refractory CLL has a median survival of 10 months. Intravenous alemtuzumab is approved in fludarabine refractory CLL. Combined alemtuzumab and fludarabine can induce responses in CLL refractory to both agents. Infusion reactions and 2-hour infusions  $3 \times$  a week are problems with IV alemtuzumab. We report on the UKCLL02 study to assess the safety and effectiveness of SC alemtuzumab in fludarabine-refractory CLL. SC alemtuzumab was given at a dose of 30 mg  $3 \times$  a week for up to 24 weeks. Patients not responding to alemtuzumab in the trial could receive oral fludarabine combined with SC alemtuzumab. 49 patients are evaluable. Responses to alemtuzumab monotherapy ( $n = 49$ ) were 7 CR (5 MRD negative, two MRD positive), 15 PR, 25 NR and two patients died on treatment. Seventeen patients (6 PRs and 11 NRs) received concurrent fludarabine and SC alemtuzumab. Two non-responders achieved a PR and one of the partial responders achieved a CR. Therefore the overall response rate for the whole cohort was 24/49 (49%) including 6 MRD negative patients (5CRs and 1 PR). 22/38 patients (58%) with poor risk deletions (11q- and/or 17p-) and/or p53 dysfunction responded to treatment. The initial alemtuzumab dose was associated with localised skin reactions in 26 patients, fever in eight and rigors in four. All reactions subsided in <48 hour. Serious infections during alemtuzumab monotherapy were: CMV reactivation (18); febrile neutropenia (10); invasive fungal infection (4); pneumonia (7) and septicaemia (2). On the combination, CMV reactivation in three cases and septicaemia in one case. All CMV reactivations resolved on antiviral therapy. Grade 3+ thrombocytopenia and neutropenia was seen in 26 and 41 patients on alemtuzumab monotherapy as well as in one and five patients on combined therapy, respectively. The median survival for campath responders was 25 months compared to 13 months for nonresponders. We conclude that subcutaneous alemtuzumab is effective in poor-risk fludarabine-refractory CLL and is well tolerated compared to IV therapy. The addition of oral fludarabine improves the response rates with acceptable toxicity.

## Poster Presentations: Cellular and Molecular Biology

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### Cryopreservation of mature monocyte-derived dendritic cells (DC) for assessment of alloreactivity in humans

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Conventional mixed lymphocyte reactions (MLR) have not been informative in predicting graft-versus-host disease. Such assays are made significantly more sensitive through the use of DCs as antigen presenting cells (APC). Human DCs may be obtained from CD34<sup>+</sup> bone marrow or blood cells, or from peripheral blood monocytes stimulated with recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and interleukin-4 (rhIL-4). Methodological difficulties exist in the use of monocyte-derived DCs, with unstable immunophenotype and reversion to macrophage characteristics on culturing in the absence of cytokines. This presents logistical problems in using DCs to assess alloreactivity. We report a method for *in vitro* generation and cryopreservation of mature monocyte-derived DCs. Peripheral blood monocytes, from healthy volunteers ( $n = 7$ ), were isolated by plastic adherence and cultured for six days in RPMI with 1% autologous heat-inactivated plasma, rhGM-CSF and rhIL-4. The DCs were matured by further culture for three days with lipopolysaccharide. Phenotypic characterisation demonstrated CD83 and CD1a expression, lack of CD14, high levels of HLA-DR, and upregulation of CD83, and co-stimulatory molecules, CD80 and CD86, on maturation. DCs were significantly more potent APCs than peripheral blood mononuclear cells in MLRs. Mature DCs were cryopreserved in 50% autologous heat-inactivated plasma, 40% RPMI and 10% dimethyl sulphoxide. On thawing, viability was  $87.6\% \pm 15.7\%$  (mean  $\pm$  SD). Following cryopreservation, CD1a, CD83 and co-stimulatory molecule expression was downregulated to the level observed in immature DCs. MHC class II molecule expression was unaffected. Despite the immunophenotypic changes, cryopreservation did not alter DC function, with no reduction in APC potency in MLRs. The MLR kinetics showed no significant difference between fresh and cryopreserved DCs. This may be explained by recovery of co-stimulatory molecule expression 48 hours after thawing. Effective cryopreservation of mature DCs will facilitate application of DC-driven MLRs in the assessment of alloreactivity in stem cell transplantation.

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### Fourier transform infrared spectroscopic studies of lymphoma, lymphoid and myeloid leukaemia cell lines

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Fourier transform infrared spectroscopy (FT-IR) is sensitive to the structural and biochemical composition of intact single cells. This study presents a novel method to characterise differences that distinguish leukaemia and lymphoma cell lines based on mathematical modelling of objective spectral measurements. Lymphoma

(Karpas), Lymphoid (REH and ACV) and Myeloid (HL60 and Megoi) cell lines obtained from the Section of Haemato-Oncology, at the Institute of Cancer Research, Sutton were studied. Cell samples were washed and diluted in normal saline. 50 microlitre aliquots were transferred onto calcium fluoride slides by cytospin to yield a monolayer. These were air dried. Spectral data in transmission mode were acquired using a Perkin-Elmer Spotlight 300 FT-IR imaging spectrometer. The data obtained for each cell line were a combination of point spectra, hyperspectral IR and white light images. Multivariate statistical techniques incorporating principal component analysis (PCA) and linear discriminate analysis (LDA) were used to construct a mathematical model. This model was validated for reproducibility. Spectra collected at different positions on the monolayer were averaged for each cell line. Our results show distinct spectral differences in the 720 to 4000 cm<sup>-1</sup> spectral region. Bands in the averaged spectra for each cell line were assigned to proteins, fatty acids, carbohydrates and nucleic acids. Pseudocolour maps generated using PCA were used to identify major differences in the spectra across each image, resulting in the clustering of cell line populations. LDA was used to maximise the separation in the model between different cell lines, whilst minimising the separation within each group. Spectral analysis by PCA and LDA demonstrated subtle variation within each cell line but revealed significant differences between cell lines and cell lineage. The FT-IR spectrum produces a molecular signature that reflects the unique set of molecular vibrations of bio-molecules present in cells. The cellular spectra reflect phenotypic differences and offers the potential to serve as a novel diagnostic technique.

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### Functional characterisation of mutations in the telomerase complex: no evidence of a dominant negative effect

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Heterozygous mutations in the RNA component of telomerase (TERC) have been identified as causing the autosomal dominant form of dyskeratosis congenita, a bone marrow failure syndrome. They have also been detected in a small subset of patients presenting with aplastic anaemia or myelodysplasia. The majority of these mutations have been found to reduce telomerase activity but do not appear to have a dominant negative effect on the activity of wild-type TERC. Using an *in vitro* telomerase assay we have investigated the functional consequences of three recently described TERC mutations: C79del, which disrupts base pairing in the pseudoknot domain and A48G and 52-55del, which are the first mutations identified that disrupt the template region of TERC. All three mutations resulted in reduced telomerase activity (<10% of wild-type TERC) when expressed alone. When each of the mutant forms was mixed with wild-type TERC, we did not observe any reduction in activity of the wild-type TERC and therefore see no evidence of a dominant negative effect. The clinical presentation of all three mutations is not atypical, with a parental generation remaining asymptomatic and for both template mutations the disease appearing as a sporadic case. It is therefore of interest that a recent report of the same template mutations suggests that they do indeed cause a dominant negative effect on the activity of the wild type enzyme (Xin *et al.* [2006] *Blood*, pre-published DOI 10.1182/blood-2006-07-035089). It is not clear why

differing results have been obtained in the two laboratories. We can only point to the fact that our data is consistent with previous reports from several laboratories indicating that the clinical manifestations of TERC mutations arise due to haploinsufficiency rather than due to a dominant negative effect.

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### Characterisation of *Hox* expression signatures during haemopoietic differentiation of embryonic stem cells

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The 39 mammalian Class I Homeobox (*Hox*) genes arranged as paralogs (A–D) in clusters (1–13) on four separate chromosomes encode master regulators of embryonic development. Aberrant expression of *Hox* genes, reported in several haematological malignancies, may reflect reactivation of dormant embryonic tissue remnants. Embryonic stem (ES) cells have the capacity to become virtually any differentiated tissue of the body and are therefore an excellent model to study the initiating processes involved in the development of malignancies. A comprehensive strategy to quantify the *Hox* signature in both pluripotent ES cells and during the critical stages of mesodermal/haemopoietic development was designed. ES cell lines were differentiated using two rounds of embryoid body (EB) formation optimised to drive mesodermal (primary EB) and then haemangioblast formation (secondary EB), followed by specific conditions to drive both myeloid and erythroid haemopoietic colony formation. Total RNA isolated from cells of each differentiation stage was converted to cDNA for *Hox* profiling by Q-PCR. Transcript copy numbers were evaluated from standard curve calculations and expression levels compared to untreated controls. Preliminary data, focussing on the *Hoxa* and *Hoxb* clusters showed that these genes were well-represented and highly expressed in the pluripotent ES cells (up to  $3 \times 10^5$  copies/50 ng RNA). A marked decrease in expression (over 70%) for the majority of *Hox* genes assayed (15/21) was observed following initiation of haemopoiesis. Only *Hoxa11* and *Hoxa13* exhibited a marked increase in expression during mesodermal/haemopoietic development and five genes (*Hoxa6*, *Hoxa7*, *Hoxb5*, *Hoxb7* and *Hoxb13*) remained unchanged. These data confirm and extend reports of *Hox* repression during the early differentiation of ES cells to committed progenitors. The mechanism of regulation of *Hox* genes in ES cells and the significance of maintenance or upregulation of a subset of genes during early haemopoietic commitment are topics for future studies.

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### Abnormal erythropoietin receptor downregulation following erythropoietin stimulation of non-small cell lung carcinoma cells

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Erythropoietin receptor mRNA and protein have been identified in a variety of cancer cell lines, as well as solid tumours. This has raised concerns about the use of erythropoiesis stimulating agents in the

treatment of cancer-related anaemia. Previously we demonstrated expression of functional erythropoietin receptors in a non-small cell lung carcinoma cell line, H838, which activated key signalling pathways in response to erythropoietin stimulation. We detected activation of STAT5, Akt and ERK which are important downstream signalling proteins in erythroid progenitors, suggesting the EpoRs in H838 cells function similarly to EpoRs in the erythroid compartment [Dunlop EA *et al.* [2006] *Neurodegen Dis* 3, 94–100.]. Recent analysis of the downregulation mechanisms operational in this cell line has highlighted a number of abnormalities. In erythroid progenitors, EpoR is downregulated by negative regulators such as SOCS3 and SOCS1 and by ubiquitination and degradation of the receptor by the ubiquitin-proteasome pathway. By inhibiting protein synthesis and analysing EpoR levels over time we found that the erythropoietin receptor is not turned over in either resting or erythropoietin-stimulated H838 cells. Additionally, we could find little evidence for ubiquitination of the receptor, although proteasome assays showed the proteasome was active. Compounding this blunted response was impaired SOCS3 induction downstream of erythropoietin signalling pathways and a delayed induction of SOCS1 when compared to the UT-7 erythroid line. Despite this impaired downregulation, there was no evidence that erythropoietin treatment increased the proliferation or invasive potential of the H838 cells. If abnormal erythropoietin receptor downregulation and degradation is a widespread phenomenon in tumour cells it could have clinical implications for those patients receiving erythropoiesis stimulating agents for cancer-related anaemia.

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### Methylation profiling of myeloid leukaemia cell lines

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Hypermethylation of CpG islands occurs during leukemogenesis particularly in AML and myelodysplastic syndrome (MDS). One of the drugs approved for the treatment of MDS is the potent DNA demethylating agent 5-aza-2'-deoxycytidine. About 30% of MDS patients with an abnormal karyotype have normalization of their karyotype after receiving the drug. However, to monitor the effectiveness of the drug, the methylation status of the genome needs to be measured. This can be done using several methods to assess the global methylation status or the methylation status of the promoter regions around candidate genes can be monitored. Pyrosequencing allows the highly reproducible quantification of methylation frequencies within individual consecutive CpG sites allowing reproducible measurement of small changes in methylation levels. This is due to the analysis of the CpG sites within the context of the surrounding DNA sequence with integrated internal controls for bisulphite conversion. In our study, we have used pyrosequencing to monitor the levels of methylation in 17 CpG sites across four genes (MLH1, p16, MGMT and LINE-1) in a panel of myeloid cell lines and a series of normal samples with the aim of monitoring methylation status of AML and MDS patients at diagnostic and during treatment. The cell lines used in the study represent a range of cell types, some with recurrent translocations. Similar levels of MLH1 methylation was seen except in Kazumi cells. However, highly variable levels of p16 and MGMT methylation, particularly U937 cells with both genes highly methylated whilst MGMT was high in K562 cells. This study will form the basis of using pyrosequencing methodology for the rapid and sensitive monitoring of clinical samples particularly to assess the effectiveness of therapeutic demethylating agents.

## Poster Presentations: General Haematology

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### Written responses to some outpatient referrals are appropriate and time- saving for patients and doctors S Gooding\*, R Cowen† and TJ Littlewood‡

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We have previously established that written responses (rather than seeing the patient in clinic) to some outpatient referrals are appropriate, safe and appreciated by general practitioners (GPs). An audit published in 2004 (Tso *et al.* [2004] *BMJ*, 329, 946–947.) showed that 103 of 104 GPs found written advice about a range of haematological problems acceptable and helpful. The audit has been repeated and included, on this occasion, a survey of patients' opinions. Eighty-five GPs, to whom a written response had been sent, were asked whether they found the written response acceptable and helpful. They were also asked whether they thought the patient was happy with the written response and whether we could survey the patient to directly ascertain their views. Of the 85 GPs, 78 responded and all found the system acceptable and helpful. All said they would be happy to have referrals dealt with in a similar way in the future. Not all GPs wanted their patients contacted but we were able to send questionnaires to 52 patients of whom 32 replied.

Twenty-nine out of thirty-two patients were aware that a referral letter had been sent. Twenty-one patients were happy with a written reply to their GP but 10 (31%) said that they would have preferred to see the haematologist in person. Twenty-five patients (78%) would have liked to see a copy of the letter sent to their GP. The data show that this system is widely appreciated by GPs but about a third of patients felt dissatisfied. We will try to address this by sending patients a copy of the letter and re-auditing. Written responses have reduced new referrals at the haematology clinic of TJL by nearly 50%. GPs are very positive about the benefits but some patients feel that they should still be seen by a specialist.

16

### A high rate of 'CLL phenotype' lymphocytes in autoimmune haemolytic anaemia and immune thrombocytopenic purpura

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The causes of autoimmune haemolytic anaemia (AIHA) and immune thrombocytopenic purpura (ITP) are unknown. About 15% of cases of AIHA and ITP are associated with underlying CLL. Conversely, about 10% of cases with CLL are complicated by AIHA or ITP. It has recently been established that clinically silent, CLL-like monoclonal B-cell lymphocytosis is considerably more prevalent than true CLL, being present in about 3.5% of healthy individuals. Together, these observations suggest the possibility that apparently idiopathic cases of AIHA and ITP are associated with subclinical CLL clones. To test this hypothesis, we investigated the presence of 'CLL phenotype' lymphocytes in 11 cases of primary AIHA, 18 ITP, two with Evan Syndrome by FACS analysis and compared with 26 age matched

healthy controls. A discrete population of stronger CD5, weaker CD20 and CD79b (over 50 events out of total 200 000 leucocytes) was classified as 'CLL phenotype' compared to normal B-lymphocytes. Six out of total 31 patients showed this subclinical 'CLL phenotype' compared to one in healthy control group, which is significant statistically (chi-square = 3.9;  $P = 0.05$ ). We speculate that these 'CLL phenotype' cells process antigen differently to conventional antigen presenting cells, thus revealing 'cryptic' epitopes, which fail to provoke previously established, appropriate tolerance or anergy, causing autoimmunity.

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### Evaluation of pneumococcal conjugate vaccine (Prevenar) in patients with myeloma and chronic lymphocytic leukaemia

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Infection with *Streptococcus pneumoniae* is responsible for significant mortality in patients with a defective humoral response. Immunisation with pneumococcal polysaccharide vaccine (PsV) is not always effective in these individuals. Conjugation of polysaccharides renders vaccines more immunogenic through T-cell dependency. Impressive results in children lend themselves to evaluation in other high-risk groups.

We assessed the immunogenicity of Prevenar in patients with myeloma and CLL. The schedule followed UK DoH guidance for vaccination of high-risk children; two doses of Prevenar followed by a dose of PsV (Group 1). Patients who had previously received PsV were only offered Prevenar (Group 2).

Data are available on 30 individuals. Twenty-four had myeloma and six CLL. Median age was 64 years (range 51–75). Six patients had never required treatment. Median chemotherapeutic treatments was two (range 0–5). Seventeen patients had undergone a transplant procedure.

Median time from last treatment was 14.5 months (range 6–69). Eight patients with myeloma were receiving maintenance thalidomide. Two had low baseline IgG levels, 10 low IgM and six low IgA.

We utilised a serotype-specific IgG Bioplex assay for all seven serotypes in Prevenar, levels greater than 0.35 µg/ml are protective.

Prior to vaccination, the median number of serotypes with protective antibody levels was 0 in Group 1 ( $n = 20$ ), (range 0–5) and 4 in Group 2 ( $n = 10$ ), (range 0–7).

Following vaccination the median number of serotypes with protective antibody levels increased to six in Group 1 (range 1–7) and a doubling of antibody concentrations was observed for a median of six serotypes (range 1–7). In Group 2 the median number of serotypes with protective levels remained at four (range 0–7) and only doubled for a median of one (range 0–7).

We conclude that the pneumococcal conjugate vaccine Prevenar appears to be immunogenic in patients naïve to PsV; however it does not benefit those previously immunised with PsV. Further studies are required prior to introduction in adult clinical practice.

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# **Treatment-related morbidity and mortality in haematology and oncology patients – a prospective survey in a district general hospital cancer unit**

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An audit was carried out in the haematology department of Ulster Hospital. The main aims of the study were to determine the risk factors for the development of neutropenic sepsis so as to derive a risk score, and audit this information against current anti-microbial protocol.

This 6-month prospective survey recorded a total of 86 episodes from February to August 2006. The majority of these episodes resulted in ward admission (60%,  $n = 52$ ), approximately half were due to neutropenic sepsis (48%,  $n = 25$ ). Among the latter group, the vast majority were aged 60 years and over (88%,  $n = 22$ ). Most of these patients also had significant co-morbidity (72%,  $n = 18$ ), abnormal liver function tests (60%,  $n = 15$ ) and a calculated creatinine clearance of less than 60 ml/min (20,  $n = 5$ ).

The most common underlying diagnosis among these patients was breast cancer (28%,  $n = 7$ ), B-cell non-Hodgkin's lymphoma (24%,  $n = 6$ ) and chronic lymphocytic leukaemia (20%,  $n = 5$ ). Most of these patients had been treated with R-CHOP21 (28%,  $n = 7$ ), FEC (20%,  $n = 5$ ) and fludarabine (16%,  $n = 4$ ). Approximately one-third despite receiving G-CSF prophylaxis presented with neutropenic sepsis (36%,  $n = 9$ ).

Septic screen revealed that almost half were culture positive (48%,  $n = 12$ ), either positive blood cultures (36%,  $n = 9$ ), urine (12%,  $n = 3$ ) or sputum cultures (8%,  $n = 2$ ). Of the positive blood cultures, the most commonly cultured organisms were coagulase-negative *Staphylococcus* (44%), *Enterococcus* (22%), *Klebsiella* (11%), *Pseudomonas* (11%) and MRSA (11%). All organisms were sensitive to Gentamycin and/or Tazocin.

The majority of patients with neutropenic sepsis made a full recovery (88%,  $n = 22$ ). The others either commenced palliative therapy or died.

Patients age 60 or over who have comorbidity and receiving chemotherapy regimen FEC, R-CHOP 21 and fludarabine (for breast cancer, NHL and CLL, respectively) are at very high risk of developing neutropenic sepsis. Hence, primary G-CSF prophylaxis is justifiable in these patients in order to reduce morbidity and mortality.

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# **Risk factors for the development of adverse gastrointestinal events post-chemotherapy**

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The Ulster Hospital delivers BCSH level 2 care for the haematology patients, and provides oncology cover for the Cancer Centre. A prospective study was conducted from February to August 2006 which aimed to assess treatment-related morbidity and mortality among haemato-oncology patients and to ascertain the risk factors for the development of adverse events.

Eighty-six episodes were recorded during this period. The main causes of morbidity were severe gastrointestinal symptoms (36%,  $n = 31$ ) and neutropenic sepsis (29%,  $n = 25$ ). Other less frequent

symptoms were suspected thromboembolism, cellulitis, headache, rash, lethargy and renal failure.

Most cases of gastrointestinal symptoms occurred in oncology patients (90%,  $n = 28$ ), including diarrhoea, vomiting, or mucositis. Most episodes were severe and resulted in ward admission (57%,  $n = 16$ ). Many patients (29%,  $n = 8$ ) had biochemical abnormalities including hypomagnesaemia, hypokalaemia, hypophosphataemia, hypercalcaemia and hypoalbuminaemia. Of the patients who were admitted, the mean duration of admission was 5.8 days (range 1–16 days). Although most patients made a full recovery (79%,  $n = 22$ ), a significant number required dose-modification or even discontinuation of their chemotherapy.

The most common underlying diagnoses were bowel cancer (50%,  $n = 14$ ) and breast cancer (48%,  $n = 12$ ). Most episodes occurred in patients aged 60 years and over (71%,  $n = 20$ ), and in those who had received previous treatment with another chemotherapy regime (61%,  $n = 17$ ). Many had significant co-morbidity (50%,  $n = 14$ ). Five patients (18%) had a creatinine clearance of less than 60 ml/min and 2 (7%) had abnormal liver function tests prior to treatment. The most common chemotherapy regimen received was FEC (25%,  $n = 7$ ), Oxaliplatin + Capecitabine (18%,  $n = 5$ ), Capecitabine only (11%,  $n = 3$ ), Taxatere (11%,  $n = 3$ ), Irinotecan + MAYO (11%,  $n = 3$ ) and MAYO only (11%,  $n = 3$ ). Other treatment included Irinotecan + Capecitabine and Vinorelbine.

Following chemotherapy, gastrointestinal adverse events are a significant cause of morbidity among oncology patients. The main risk factors are: age 60 years or over, underlying diagnosis of bowel or breast cancer, treatment with FEC or Capecitabine, co-morbidity and abnormal liver function tests.

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# **Survival of patients with haematological malignancy admitted to the intensive care unit (ICU): prognostic factors and outcome compared to unselected medical ICU admissions**

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Historically, cancer patients had a poor outcome following ICU admission. We assessed survival and factors predicting survival for patients with haematological malignancy to improve ICU patient selection. We also assessed the APACHE II scores ability to predict outcome for these patients. One non-surgical admission within  $\pm 1$  week of each hematological admission acted as a control group. Factors assessed by multivariate regression analysis were age, diagnosis, time from haematological diagnosis to ICU admission, degree of prior treatment, remission status, prior stem cell transplant, documented infection and length of neutropenia. For haematology patients, predicted hospital mortality was calculated from the APACHE II score. We identified 111 patients with haematological malignancy (acute leukemia  $n = 42$ , chronic leukemia  $n = 11$ , myeloma  $n = 19$  and lymphoma  $n = 39$ ) admitted to ICU in four hospitals (November 2000 to January 2006). Haematological patients median age was 59 years (range 17–84), M:F ratio 1.22:1. Control patients ( $n = 111$ ) were similar: median age 63 years (range 17–86), M:F ratio 1.09:1. For control patients, overall ICU and hospital survival rates were 70% and 55%, respectively, while survival for haematology patients was approximately half at 44% and 24%,



respectively. In multivariate regression analysis, only increasing age ( $P = 0.016$ ) and documented infection ( $P = 0.016$ ) predicted poor outcome. APACHE scores were significantly higher in haematology patients (median 27) than controls (median 19)  $P < 0.001$  (two-sample  $t$ -test). Haematology patients predicted hospital mortality (56%) was significantly lower than actual mortality (77%)  $P < 0.001$  (one-sample test of proportion). For controls, hospital survival was slightly reduced if mechanical ventilation (MV) required (risk ratio = 1.37; 95% CI = 0.91, 2.05). Haematology patients hospital survival was significantly worse for MV – 5/55 (9%) vs no MV 20/44 (45%) (risk ratio = 5.00; 95% CI = 2.04, 12.50). Most pre-admission variables assessed did not predict mortality and should not be used for this purpose. In haematological malignancy, need for MV still predicts poor outcome but without MV nearly half survive to hospital discharge.

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### **A single centre study of the acute pain experienced by adult patients undergoing a bone marrow procedure** **L Lloyd**

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**Background:** Bone marrow aspirate/trephine procedures (BMAT) are performed in a wide variety of patients. Research suggests that acute pain is an integral part of BMAT despite administration of Lidocaine pre-procedure. Once diagnosed with a bone marrow disorder patients are often required to endure multiple BMAT. Patients who experience severe pain may become anxious and afraid of having subsequent tests. Provision of adequate analgesia, sensory and procedural information and priming patient expectations are recognised as key factors that positively impact on the patient's pain experience. There is, however, a dearth of information regarding the quantity and quality of pain associated with BMAT.

**Aim:** The study's primary aim was to assess the intensity of acute pain experienced by adult respondents undergoing BMAT in order to evaluate efficacy of Lidocaine. It's secondary aim was to identify sensory and affective words associated with BMAT as a means to improve future patient's experience.

**Methodology:** Fifty-six consecutive patients were recruited over a six month period (34 men [61%] and 22 [39%] women). The data collection tool was the Short Form – McGill Pain Questionnaire incorporating a Visual Analogue Scale [VAS] and a present pain index. Data analysis was performed using descriptive statistics, paired  $t$ -test, McNemar test, Fischer exact test and logistic regression techniques.

**Results:** Twenty-four respondents (43%) reported a VAS pain rating of 4 or above with 25% of respondents scoring between 5.4–9.9. The mean VAS pain intensity score was 3.7 cm (range 0–10).

The most frequently selected quality words chosen by respondents to describe the pain experienced during the BMAT procedure included; sharp (45%), shooting (36%), aching (35%), tiring-exhausting (17%) and fearful (15%).

**Conclusion:** Lidocaine did not provide adequate pain relief. Provision of supplementary analgesia, alongside procedural and sensory information may help to improve the patient experience. We propose repeating the study utilising Entonox.

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### **Interim development report from the UK NEQAS Haematology Web Based Digital Morphology Pilot Scheme, registered for continuing professional development (CPD) with the Institute of Biomedical Science (IBMS)**

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UKNEQAS(H) in collaboration with Manchester Royal Infirmary and Manchester Universities have introduced a Web based pilot scheme for Digital Morphology, registered with the IBMS for CPD. In April 2005 participating centres of the conventional Morphology Scheme were invited to register one individual with UKNEQAS(H). In April 2006 registration was increased from 221 to 412 individuals, from 14 countries (>85% UK).

Two Web based cases were released quarterly (seven releases), consisting of multiple digital images from previous UKNEQAS(H) morphology surveys with coded comment report sheets and reflective feedback forms, 1CPD point was awarded per completed case.

Exercise completion by registrants was 51% (43–69%). The majority of participants (72%) spent <30 mins reviewing cases, with additional time background reading. Cases included Haemolytic conditions and Leukaemias. Of those who commented >70% stated an improved awareness of the clinical conditions, <20% stated that their knowledge had not changed (variation was associated with the clinical condition).

Feedback was used to develop the scheme format; optical magnifications now appear with images and the clinical data streamlined. Image quality has improved and criticism of red cell images used to progress the project. Comments included: raised awareness of Haematology, improved understanding of clinical features and laboratory results (cell markers, cytogenetics, chemistry) and of specific morphology (significance of granulation or nuclear appearance).

Participants stressed the usefulness of images for teaching, particularly for rare haematological cases seen less frequently in some laboratories. Future development by UKNEQAS(H) include a revised protocol for reflective reporting, introduction of electronic reporting and improved access to viewing software for larger images.

The scheme's aim remains one of education, rather than assessment, directed at individuals rather than centres. With the key theme of personal professional development promoting improvement to the quality of haematological morphology this scheme has potential for expansion across the UK and internationally.

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### **Non adherence to dosing guidelines for LMWHs in renal impairment leads to a high incidence of significant bleeding**

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**Introduction:** Recently Guidelines have been developed for dose reduction in LMWHs in the treatment of patients with renal impairment. Following a number of significant bleeding events

related to LMWH (Enoxaparin) usage in Medical admissions to this hospital we prospectively audited Enoxaparin usage in patients with renal impairment and the incidence of adverse events over a three month period. All patients included were receiving therapeutic doses of Enoxaparin for the treatment of thromboembolic events or ischaemic heart disease.

**Methods:** Estimated GFR (eGFR) was calculated using the four variable MDRD formula. Twenty patients with an eGFR <30 ml/min/1.73 m<sup>2</sup> who were commenced on a therapeutic dose of Enoxaparin over the three month period were included. Patients were assessed as to whether they were weighed prior to commencing treatment and if Enoxaparin dose was reduced appropriately as per established guidelines. The number of adverse bleeding events was recorded. Antifactor Xa levels were measured on each patient. Concurrent antiplatelet drug usage was also recorded.

**Results:** The mean age of the 20 patients included (nine male, 11 female) was 75. Fifteen out of twenty (75%) patients were incorrectly prescribed a higher initial dose than appropriate based on their weight and renal function. Of these patients 5/15 (33%) developed significant GI haemorrhage. One patient developed a haematoma at the injection site. Of the six patients who developed bleeding complications five had therapeutic Antifactor Xa levels and only one had an elevated level > 1 u/ml. Eleven out of twenty (55%) patients were commenced on initial treatment without a recorded weight.

**Conclusions:** Failure to record weight and to appreciate renal impairment can lead to excessively high dosing of LMWHs in patients admitted to general medical and cardiology wards. Renal function frequently changes over the duration of medical admissions and the dose of LMWHs should be reviewed daily and altered accordingly in order to avoid serious complications. Routine measurement of Antifactor Xa does not identify patients at risk of bleeding and should not be routinely measured.

## 24

### A funding framework for high cost haematology drugs in Merseyside and Cheshire Cancer Network (MCCN)

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**Introduction:** Cancer chemotherapy drug costs are excluded from the national tariff. There is considerable delay between drug licensing and National Institute of Clinical Excellence (NICE) appraisal and not all drugs are appraised. Haematology Services are funded through the commissioning of local hospital services by Primary Care Trusts (PCTs). This has meant that some high cost drugs have not been available to all patients. We describe the development and operation of a new high cost drug framework (HCDF) for cost per case funding haematology drug treatments.

**Methods:** The network haematology group agreed a standard protocol framework for some high cost therapies. An Effective Therapies paper, stating the clinical evidence for each treatment, its place in practice and expected cost per 100,000 population for the current financial year was approved by the local specialist and collective commissioning group (LSCCG) and circulated to all Primary Care Trusts. MCCN also developed a standardised case of need pro-forma and robust centralised pathways for funding requests.

**Results:** During the first 6 months of the framework, MCCN received 35 requests for funding. Thirty (85%) of these were for MCCN cancer patients. Of these 27 (90%) cases were approved and three (10%) were not. Twenty-six out of twenty-seven (96%) requests within the

framework were approved. Only one of three (33%) requests outside the HCDF was approved. Sixteen out of thirty (53%) of all requests were for Bortezomib. There were two requests each for Alemtuzumab and Depocyte (Liposomal Cytarabine) and Neulasta (Pegfilgrastim). **Conclusions:** A high cost drug framework consisting of selected effective therapies and robust cancer network pathways is an effective way of securing funding for previously unfunded high cost haematology cancer drugs. The cancer network also holds previously unavailable high cost drug funding data.

## 25

### Pseudo-hyperkalaemia and aetiology of thrombocytosis: a six-year retrospective correlation study

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Pseudohyperkalaemia is a rarely encountered event that causes unnecessary anxiety among clinicians, and may lead to inappropriate treatment to lower a spuriously raised potassium level. The association between pseudohyperkalaemia and aetiology of thrombocytosis is unclear. A 6-year retrospective audit was conducted on 90 patients with thrombocytosis referred to the Haematology Department in Ulster Hospital Dundonald, a large district general hospital. Over two thirds of this study population had myeloproliferative disorders, and the most common diagnosis was primary thrombocythaemia (41%,  $n = 37$ ). In contrast, reactive thrombocytosis was observed in approximately one third of the cases (32.2%,  $n = 29$ ). Pseudohyperkalaemia with apparent potassium level above the upper limit of the normal range was observed in the majority of patients with thrombocytosis from any aetiology (60%,  $n = 54$ ). The likelihood of finding pseudohyperkalaemia was highest among patients with primary thrombocythaemia (75.7%,  $n = 28/37$ ), followed by polythaemia rubra vera (75%,  $n = 12/16$ ), and reactive thrombocytosis (34.5%,  $n = 10/29$ ). A significant positive correlation was observed between the platelet counts and the serum potassium level (Spearman's correlation coefficient,  $r = 0.28$ ,  $P = 0.01$ ).

## 26

### Workshop on developing digital images of blood and bone marrow morphology for external quality assurance led by UK NEQAS for General Haematology

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A Web based Scheme for Digital Morphology, registered with the Institute of Biomedical Science for CPD was successfully introduced in 2005 by UKNEQAS(H), in collaboration with Manchester Royal Infirmary and Manchester Universities. A workshop was held for invited clinicians and scientists to assist in prioritising development projects in digital morphology.

Delegates were sent two unstained blood smears to be stained locally, these were returned for imaging at MRI and assessed at the workshop. Delegates were required to complete a questionnaire and

view two virtual slides, composed of multiple high power images, depicting a bone marrow smear of acute leukaemia and a blood smear of plasmodium falciparum.

The workshop formed two focus groups, the Biomedical Scientists considered the expansion of the digital CPD scheme, the Clinical Haematologists considered issues around digital morphology for assessment and teaching.

Review, by the workshop, of images highlighted the variety in range and quality of staining techniques nationally which requires addressing before definitive imaging standards can be achieved. Expectations of image quality and content varied and the need to standardise image viewing software was highlighted.

Both focus groups identified future roles for images in education and assessment, for both organisations and individuals, including the possible use of images in performance monitoring at different levels.

UKNEQAS(H) has a major role in the assurance of performance quality. Participants of Schemes must help lead the way in development by providing critical feedback. The workshop produced intense debate on how emerging web based technology should be used to not only educate but possibly to assess performance in morphology both for centres and individuals. The promotion of personal professional development by improvement to the quality of haematological morphology in different professional groups was denoted as of immense importance. Workshop feedback will be used to develop digital morphology in EQA.

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### **JAK2 mutation in the diagnosis and management of myeloproliferative disorders**

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According to the World Health Organisation classification, myeloproliferative diseases encompass polycythaemia vera (PV), essential thrombocythaemia (ET), myelofibrosis (MF), and other less well-known disorders.

In 2005, several papers were published which reported an association between the myeloproliferative disorders and an acquired, single-point mutation (valine to phenylalanine) in the JAK2 gene. In one published series the mutation was detected in 65–97% PV patients, 23–57% ET patients, and 35–57% MF patients.

This study was undertaken to investigate these findings in our own patient population. Case notes were examined for haematological profile at diagnosis, haemorrhagic and thrombotic episodes, venesection frequency, hydroxycarbamide use, and spleen size. Bone marrow trephine biopsies, along with haematological parameters, were reviewed to confirm the diagnosis. A PCR method was utilised to detect the presence of the JAK2 mutation in whole blood samples. JAK2 mutation tests were undertaken in 70 patients with possible myeloproliferative disorders attending haematology outpatient clinics over a 3-month period. Fourteen (20%) were secondary polycythaemias and were excluded. Of the remaining 56, 31 (55%) were PV, 21 (38%) were ET, and 4 (7%) were MF. JAK2 mutation was detected in 25 (81%) PV cases, 11 (52%) ET cases, and in 1 (25%) MF case. Thrombotic complications were identified in 8 (22%) JAK2-positive patients, vs 5 (26%) JAK2 negative patients. Haemorrhagic complications were found in 14 (38%) JAK2 positive patients, compared to 2 (11%) of those lacking the mutation. The average maintenance dose of Hydroxycarbamide was lower (554 mg/day vs 861 mg/day) for JAK2-positive patients.

Our data mirrors the findings of the recent studies in terms of the overall frequency of the JAK2 mutation. Notably, our study indicates that patients possessing the aberrant form of JAK2 are more sensitive to hydroxycarbamide. JAK2 mutation testing is a straightforward and useful diagnostic tool in the myeloproliferative disorders and is valuable in subsequent management.

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### **How good is documentation of telephone advice given by haematologists?**

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It is important that medical notes are completed accurately and fully for patient safety, resource use and medico-legal reasons. Haematological advice is commonly given by telephone. We observed that this was variably recorded in hospital medical notes and decided to undertake a formal assessment.

An audit of telephone advice given over a 9-week period was conducted. The haematologist recorded the advice given and the medical notes were later reviewed to ascertain whether the advice given was documented correctly. Advice given to general practitioners was not recorded due to the practical difficulties associated with reviewing records in the community. A total of 48 episodes were recorded. The majority of advice was given to general medical teams (22), followed by neurosurgery (7), neurology (6), orthopaedics (4), general surgery (3), paediatrics (3), A&E (2), ITU (1). Telephone consultations were given for the following areas: clotting (20), general haematology (18), transfusion (8) and malignant haematology (5). The commonest questions were regarding thrombocytopenia (8), and abnormal clotting results (10). Only 27/48 (56%) of consultations were documented accurately in the medical notes. Eleven out of forty-eight consultations were not documented at all and 10/48 consultations were incompletely documented.

These findings indicate that recording of telephone advice is poor. This means that members of the medical team reviewing a patient at a later time may not be aware of the advice given and may lead to inappropriate investigation or intervention. It also creates a potential medico-legal risk. It is not usual practice for haematologists to keep their own record of advice given or arrange to write in the notes themselves. We suggest those giving telephone advice should consider either specifically requesting that the discussion is documented in the patient's medical notes or recording the consultation themselves.

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### **Natural history and outcome of amyloidosis in the elderly**

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The natural history and outcome of elderly patients with systemic amyloidosis has been little studied. Three hundred and ninety-two patients over age of 75 years were referred to our centre between 1988 and 2006, amongst whom amyloidosis was confirmed in 292 cases. One hundred and eighty-three had systemic AL type, 38 had localised AL, 30 had AA amyloidosis, 21 had a type of hereditary amyloid, 7 had B2 M, and 13 had wild-type ATTR. Median overall survival (OS) of the cohort was 12 months. OS was 12 months in AA, 9 months in

AL, 33 months in hereditary types, 22 months in B2 M, and 16 months in wild-type ATTR. Various chemotherapy regimes were used in AL, most frequently oral melphalan and prednisolone; OS was significantly better among treated patients than those who did not receive chemotherapy (19 vs 7 months,  $P < 0.0001$ ). The prognosis of AA and

AL amyloidosis is poor in the elderly, but many patients with AL were able to tolerate chemotherapy and achieve a clonal disease response that may have improved their outcome. These observations support trialling newer, more rapidly acting chemotherapy regimes in the elderly population.

## Poster Presentations: Haemostasis and Thrombosis

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### Implementing an external quality assessment programme for users of CoaguChek XS and CoaguChek XS Plus for monitoring INRs

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Using lyophilised plasma, UK NEQAS Blood Coagulation has provided External Quality Assessment (EQA) programmes for INR both in the laboratory and for Point of Care (POC) devices for many years. Currently the POC INR programme has 930 participants using CoaguChek or CoaguChek S (the most commonly used POC INR devices in the UK).

Two new devices – the CoaguChek XS (CUC XS), for patient use, and the CoaguChek XS Plus (CUC XS Plus), for healthcare professional use, were launched in 2006. In clinical practice both measure INR in whole blood samples. Introduction of these devices has demanded formulation of new material for EQA. In a pilot exercise, two lyophilised plasma samples (samples 1 and 2) plus reconstitution and recalcification fluids were distributed to 23 centres routinely using CUC XS or CUC XS Plus. Results showed good precision; sample 1 – CUC XS median INR = 3.0, CV 8%, CUC XS Plus median INR = 3.2, CV 6%; sample 2 – CUC XS median INR = 3.45, CV 4%, CUC XS Plus median INR = 3.4, CV 6%. In this first exercise, 30% of tests resulted in an error code (no result). In a second exercise, 2 lyophilised plasmas (samples 3 and 4) prepared using a modified method were distributed. Precision was again good; sample 3 – CUC XS median INR = 1.5, CV 4%, CUC XS Plus median INR = 1.6, CV 18%, sample 4 – CUC XS median INR = 3.05, CV 3%, CUC XS Plus median INR = 3.0, CV 6%. The error rate (8%) for samples 3 and 4 was improved. The small differences between median INRs with CUC XS and XS Plus were not significant. In conclusion, EQA for CoaguChek XS and CoaguChek XS Plus is possible. UKNEQAS BC will formally launch an EQA programme for these devices during 2007.

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### Assessment of risk and prophylaxis for deep venous thrombosis and pulmonary embolism in medicine wards and ICU in a developing country

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Deep vein thrombosis (DVT) and pulmonary thromboembolism (PE) are important causes of morbidity and mortality in hospitalized patients. This study was done to assess risk factors and prophylaxis given for DVT and PE in medically ill patients using risk stratification score card.

An observational study on 117 patients over 9 months was done in medicine ICU and wards. Structured proforma was designed using a standard protocol and effective risk stratification for DVT was done

in low, moderate, high and highest categories. Patient was followed up for a week to record any changes in the risk categories and document any signs of PE or DVT if present.

Sixty-eight patients (58.1%) belonged to the wards and 49 (41.9%) to the ICU. Patients with respiratory illness contributed 37.6% of sample size. Ninety seven (82.9%) patients had high to highest risk for DVT and PE, out of which only 11 (9.4%) got DVT prophylaxis and D-dimer and Doppler were done only in 6 (5.13%) patients. On follow up over 7 days, 36 (30.8%) patients got discharged and 19 (16.2%) patients died. Of the remaining 62 patients, 48 (77.4%) belonged to high to highest risk category. Clinical signs and symptoms of DVT were present in 19 (30.6%) while that of PE was seen in 6 (11.1%).

Seventy-one per cent of medical ward patients and 100% of ICU patients observed during the course of study had high to highest risk for DVT and PE, but only 16.33% in ICU and 4.41% in wards got prophylaxis. Investigations for confirming DVT and PE were done only in 5.13% patients. Thus significant risk for DVT and PE exists in medical patients admitted to wards and ICU but only a small proportion of the patients are given prophylaxis or investigated. This study underlines the need to aggressively implement DVT risk stratification strategy in medical patients and provide prophylaxis unless contraindicated.

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### Observational study of clinically suspected heparin induced thrombocytopenia (HIT) at the Royal Cornwall Hospital – the value of pre test probability score and nature of our patient population

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Heparin-induced thrombocytopenia (HIT) is an immune-mediated prothrombotic condition caused by the anticoagulant heparin. It is considered to be a clinicopathological syndrome as the diagnosis is based on clinical and laboratory findings.

A scoring system known as the 4 Ts (Thrombocytopenia, Timing of platelet count fall, Thrombosis, other causes of Thrombocytopenia) has been devised to estimate the likelihood of HIT.

We retrospectively reviewed 27 cases of clinically suspected HIT. We looked at the clinical history and assigned a pre test probability score to each case. The HIT antibody test results were noted.

Sixty-seven per cent of cases were medical patients and only 15% were surgical patients. This is interesting as published data suggests that the incidence of HIT is higher in surgical patients.

Forty-eight per cent (13/27) of patients with suspected HIT were given heparin for thromboprophylaxis whilst 41% (11/27) of patients were given higher doses of heparin for treatment of venous thromboembolisms and acute coronary syndrome.

Seven out of twenty-seven suspected HIT patients had confirmed HIT.

Sixty-seven per cent of those patients were treated with unfractionated heparin.

PreTest Probability (PTP) scoring) was found to be strongly correlated with positive HIT antibody test (ELISA) for clinically significant HIT.

One hundred per cent of patients with high PTP score and 40% of patients with intermediate PTP score with none of the patients with low PTP score were HIT test positive.

Low PTP scoring for HIT seems to be suitable for ruling out HIT in most clinical situations which means PTP has a high negative predictive value.

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### **Inadequate clinical information during the day makes warfarin dosing perilous by on call medical staff during the night. Time to change the anticoagulation prescription chart?**

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With an increasing elderly population in atrial fibrillation the numbers of patients taking warfarin continues to rise. Warfarin is more effective than aspirin in reducing the risks of a thrombotic stroke in these patients. Warfarin dosing can be difficult in the elderly related to defective liver metabolism, dietary vitamin K deficiency and polypharmacy with potentiating drugs. INR results tend to arrive late in the day hence warfarin dosing for inpatients is primarily undertaken by the on call medical team rather than the patient's normal treating team. This should not be a problem if there is adequate information relating to the patient's indication for warfarin, normal maintenance dose and target INR. Frequently this information is defective compromising care. To assess the scale of the problem we undertook an audit of patients receiving warfarin who were admitted as medical emergencies to this hospital on a single day and monitored them whilst they remained inpatients. This group comprised 27 adult patients. Twenty-one were already on warfarin and six were started on warfarin from scratch. Reviewing the case notes the clinical indication was available in 44%, usual warfarin dose available in only 26% and target INR available in 52%. Not surprisingly with this lack of clinical information there were significant problems with warfarin prescribing by the on call teams meaning that non-therapeutic INRs occurred in 58%. One patient in particular became grossly overanticoagulated because the on call team restarted warfarin as per the standard anticoagulation protocol and did not consider the fact that the patient only took 1mg of warfarin daily. Thankfully there were no bleeding complications. Following this audit we have proposed some simple alterations to the anticoagulation prescription chart improving the available clinical information that should help to prevent inappropriate warfarin dosing in the future.

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### **What is the role for baseline coagulation screens in acutely ill patients admitted to the medical assessment unit?**

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We retrospectively audited the results of baseline coagulation screens performed on 100 acutely ill patients admitted to the medical assessment unit during a 2-week period. We wanted to determine

whether this was an appropriate use of resources and how the results affected clinical care. Coagulation screens were performed on 53/100 patients. The group comprised 35 females and 18 males, with an age range of 23 to 91 (mean 64). Six out of fifty-three samples were underfilled or haemolysed and although requests were made directly to the medical team for further samples none were received. Indications for the screen were diverse and the commonest was confusion. Only 9/53 were clinically appropriate based on well established criteria such as patients with acute bleeding. The abnormal pickup rate in this group was 5/9 (55%) and all these results were clinically significant directly affecting patient care. Of the 44 inappropriate requests the abnormal pickup rate was only 7/44 and clinically significant results only amounted to 2/44 (2.2%). We were able to highlight a problem with the medical assessment unit nursing staff performing a range of screening blood tests on the majority of patients before being seen by the on call medical team. Apart from the cost a major problem with performing indiscriminate coagulation screens was the time factor on our haematology biomedical scientists who are already overstretched with all the other demands of the on call service. Our audit highlighted the need for education and the use of protocols to guide investigation. We felt that with such protocols we could reduce coagulation screen requests by over 80% on the medical assessment unit and when one considers that a coagulation screen costs £6.50 this could amount to significant savings for the trust especially if this change of practice could be extrapolated to other departments.

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### **Problems with relying on the PFA100 as a screening test for platelet function disorders**

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The PFA100 analyser is a useful tool in the initial assessment of patients with possible von willebrand's disease or a platelet function disorder. Invariably these patients are referred to the haematology department because of a history of abnormal bruising, mucosal bleeding and immediate bleeding after surgical procedures. They may also be referred because of family screening. When considering a possible bleeding diathesis it is paramount to take a detailed bleeding history beforehand in order to guide investigations. The PFA100 is thought to have a sensitivity of greater than 95%. Generally patients with platelet function disorders will have a prolonged collagen-epinephrine plus collagen-ADP closure times. An isolated prolonged collagen-epinephrine closure time is normally associated with the effects of anti-platelet drugs like aspirin. During the past nine months we have been investigated four patients who had abnormal mucosal bleeding and an isolated prolonged collagen-epinephrine time. All avoided aspirin plus non-steroidal anti-inflammatory drugs. The group comprised two females and two males. Platelet aggregation tests fitted with a probable platelet release defect in two and a platelet storage pool disorder in the other two. During the same time period we have also seen six other patients, five females and one male, with entirely normal PFA100 results but probable platelet release defects based on aggregation studies. The explanation in two probably related to having supranormal levels of von willebrand's factor. These cases illustrate the fact that in patients with a convincing history of mucosal bleeding formal platelet aggregation studies are appropriate even if the PFA100 results are normal and an isolated prolonged collagen-epinephrine closure time

does not necessarily just mean the effects of anti-platelet drugs. With such variable PFA100 results in patients with mucosal bleeding and normal von willebrand factor results should we stop doing the PFA100 and just go straight to aggregation studies?

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### **An audit of the molecular service for the identification of mutations in type 2 VWD**

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In 2003 molecular testing for the detection of mutations in type 2 VWD was set up in our molecular haemostasis laboratory. To date, 38 patients have been investigated. Based on the phenotypic results and/or the clinical details provided, 29 (76%) had qualitative defects (13 type 2N and 16 type 2A/B/M) and nine (24%) had quantitative deficiency. The patients with type 2N and quantitative VWD will not be discussed in this abstract.

In the remaining 16 patients, 15 (94%) had discrepant VWF:Ag and VWF:Act (VWF:RCO and/or VWF:CB) results with ratios  $<0.7$ ; the remaining patient had levels all  $<5\%$ , so discrepancies were hard to distinguish. In 10/16 (62.5%) patients VWD mutations were found; a further patient was later found to have platelet-type pseudo VWD (p.Gly249Val). Of the ten qualitative mutations found, two were type 2M (p.Arg1315Cys and p.Arg1426Cys), two were VWD Vincenza (p.Arg1205His), three were type two unclassified (p.Arg1374Cys), two were type 2B (p.Arg1306Trp and p.Val1316Met) and one was type 2A (p.Ile1628Thr). Seven out of ten had VWF levels measured more than once before referral; two of the remaining three were related to a patient tested more than once and in whom a mutation had been identified. In the five patients where no mutation was identified, all had only one discrepant VWF activity assay (RCO/Ag = 3 and CB/Ag = 2) and 3/5 only had their VWF levels measured once suggesting the discrepant result could be an outlier.

Overall, mutations were found in approximately 70% of cases, when the platelet-type pseudo VWD case is included. In patients who had VWF levels tested more than once, a mutation was identified in 80% highlighting the importance of performing phenotypic assays more than once to confirm the disease and potential subtype before referral. In conclusion, we believe this audit has shown that the method developed in our laboratory for detection of mutations in type 2 VWD is appropriate for investigation of patients with these qualitative defects.

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### **Acquired haemophilia in a district general hospital – 18-year lookback**

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Current literature on acquired haemophilia A mainly consists of patients referred to tertiary referral centres. This may result in a reporting-bias, not accurately reflecting the true features of this disease. This report looks at a consecutive, unselected cohort of patients presenting with acquired haemophilia to the Royal Cornwall Hospital between 1988 and 2006. A total of nine patients were reported, giving an incidence of 1.43/million/year. Compared to previously reported cohorts, patients were older (median age 77 years), and more likely to have an underlying diagnosis (67%). The bleeding phenotype was less severe than in much of the current literature with 33% of patients not requiring haemostatic treatment and not a single case of a fatal bleed. Response to immunosuppression

was lower than in some recent reports, with 63% of treated patients recovering a normal FVIII level and an undetectable inhibitor. Out of the patients studied 56% died, though in no case was death attributed to the presence of an inhibitor. There was significant morbidity associated with immunosuppressive treatment however. This suggests much of the current literature based on data from referral centre patients may not be applicable to patients with acquired haemophilia A presenting to other centres who are likely to be older, have milder bleeding and be less able to tolerate immunosuppression.

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### **Source and use of reference ranges in haemostasis: practice amongst UK NEQAS for Blood Coagulation participants 2005–6**

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Some guidelines on interpretation of haemostasis tests and assays recommend use of locally determined reference ranges to optimise detection of haemophilic and thrombophilic defects. However, access to adequate numbers of appropriate donors to allow construction of local reference ranges is problematic. In two separate questionnaires, UK NEQAS for Blood Coagulation participants were asked for the source of their reference ranges for thrombophilia testing (May 2005) and routine screening tests and factor assays (March 2006). The findings are summarised below.

For the prothrombin time and APTT, 70% of centres employ a locally determined reference range. However, only 57/202 constructing local reference ranges include 40 or more individuals in the reference population. Laboratory staff form the largest group of donors (85/210). The second largest group ( $n = 36$ ) is identified as 'normal patients'. Only 37% of laboratories performing Clauss fibrinogen assays construct a local reference range, with 37% using the range suggested in the manufacturers' literature. For factor assays the percentage of laboratories determining local reference ranges falls to an average of 22%. The remainder use either manufacturers data (42%) or peer-reviewed literature (34%). For some assays participants using manufacturers data or peer-reviewed literature quoted clearly inappropriate reference ranges, for example 152 centres reported a lower limit for factor XI:C of 50 U/dl, despite bleeding episodes reported in subjects with heterozygous deficiency and levels above this value.

For thrombophilia testing, it is only for PS antigen measurement that the majority of centres construct a locally determined reference range; for free PS antigen 23/57 of these centres employ a gender-specific range. For some assays, centres employing kits from the same source and quoting the manufacturer as a source of reference range reported different ranges.

Difficulty in establishing appropriate reference ranges to ensure accurate diagnosis of haemostatic defects is a challenge to laboratories; These data confirm the variable practice employed.

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### **Soluble CD40 ligand and atrial fibrillation: relationship with platelet activation and endothelial damage/dysfunction**

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Increased levels of soluble CD40L (sCD40L) in the plasma are present in many cardiovascular diseases and predict a poor outcome

in long-term follow-up studies. Levels in atrial fibrillation (AF), a leading cause of thrombotic stroke, are unknown. The precise source of this molecule is unclear and although the platelet is frequently cited as the source of sCD40L, this is not universally recognised. We hypothesised (a) raised levels of sCD40L in non-rheumatic AF, and (b) that levels sCD40L correlates with platelet, but not endothelial, markers, thus suggesting a platelet origin.

sCD40L, platelet marker soluble P selectin and endothelial markers von Willebrand factor and soluble E selectin were measured by ELISA in the plasma of 54 AF patients free of diabetes or major cardiovascular disease, and in 28 age- and sex-matched controls in sinus.

Median (inter-quartile range) sCD40L in AF was 0.82 (0–4.8) ng/mL compared to 0.21 (0–5.5 ng/mL) in controls ( $P = 0.0397$ ). Both von Willebrand factor and soluble P selectin were also raised ( $P < 0.005$ ) in AF, but none of the three indices inter-correlated significantly. Soluble E selectin was not significantly elevated in AF.

sCD40L is marginally raised in AF but the stimulus for this is unclear. The lack of clear correlation with relevant plasma markers suggests that the source is unlikely to be the endothelium or platelet alone.

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ABSTRACT WITHDRAWN

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# **Anticardiolipins and pregnancy outcome: a retrospective audit**

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Elevated anticardiolipin (ACA) levels are associated with increased risk of adverse pregnancy outcome. In a retrospective audit we investigated whether the class and/or titre of ACA (IgG or IgM) affected pregnancy outcome and examined the effect of treatment with low-dose aspirin and/or Low Molecular Weight Heparin (LMWH) on outcome.

From April 2004 to March 2006, 175 pregnancies in 73 women with IgG > 9 GPL, or IgM ACA > 11 MPL and no other thrombophilia were included in this study. Sixty-nine (39%) and 106 (61%) of the pregnancies respectively, occurred in women with elevated IgG or IgM ACA. Seventy pregnancies were treated with aspirin and/or LMWH. Main outcome measures were preterm delivery (PTD), growth restriction (IUGR), recurrent (> 3 consecutive) first trimester (T1) miscarriages and 2nd (T2) or 3rd (T3) trimester intrauterine death. Outcomes were compared using chi-square test.

For both classes of ACA, there were significantly more adverse outcomes in the untreated groups ( $P < 0.05$ ). There was no significant difference in total adverse outcomes in the untreated IgG vs IgM groups, except recurrent 1st trimester miscarriage where elevated IgG was more significant ( $P = 0.05$ ). Four patients, nine pregnancies, had

ACA level > 40 GPL or MPL – 6 untreated miscarried, two were delivered prematurely after treatment, and one normal delivery was complicated by maternal thromboembolism.

Pregnancy outcome is significantly worse in untreated ACA-positive women, even if the levels are lower than the diagnostic criteria for antiphospholipid syndrome (> 40 GPL/MPL). Consideration should be given to treating all pregnant women with weakly positive ACA.

Patient group	Normal outcome	Total adverse	IUGR	PTD	T1	T2	T3
IgG treated	12 (48%)	13 (52%)	1 (4%)	3 (12%)	9 (36%)	0	0
IgG not treated	10 (23%)	34 (77%)	0	0	29 (66%)	2 (5%)	3 (7%)
IgM treated	26 (58%)	19 (42%)	7 (16%)	4 (9%)	8 (18%)	0	0
IgM not treated	20 (33%)	41 (67%)	4 (7%)	4 (7%)	29 (48%)	3 (5%)	1

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# **Incorporation of human microvascular endothelial cells into the whole blood thrombin generation assay** **KA Tappenden\*,†, G Evans†, MJ Gallimore†,‡, IJ Mackie§, HP Wendel¶, M Winter† and DW Jones\*,†**

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There is considerable interest in assays that reflect TG in as near to physiological conditions as possible. We have developed a whole blood (WB) thrombin generation (TG) assay that has been adapted to include human microvascular endothelial cells (HMVEC) in an attempt to mimic the haemostatic role of the healthy vascular endothelium. The cellular TG assay was performed on eight healthy control WB samples in the absence and presence of HMVEC. Whilst there was no significant difference between peak height (PH) values in the absence or presence of HMVEC (range 140–328 and 135–338 nM, respectively), the endogenous thrombin potential (ETP) values in the absence or presence of HMVEC (range 1586–3541 and 946–3675 nM min, respectively) were significantly lower in the presence of HMVEC ( $P = 0.02$ ). Protein C was included in the cellular TG assay ( $n = 3$ ) to give final concentrations of zero, 3, 6 and 9 µg/ml of WB in the presence and absence of HMVEC. In the absence of HMVEC the pH remained constant between 0 and 6 µg/ml protein C (mean = 223 nM, SD ± 14.6). At 9 µg/ml the PH increased to 258 nM (SD ± 21.2). The ETP values were not affected by increasing protein C concentrations (mean = 2478 nM min, SD ± 174.5). In the presence of HMVEC there was a reduction in PH and ETP values from 208 to 137 nM and from 2245 to 1452 nM min respectively between 0 and 6 µg/ml protein C. With 9 µg/ml protein C, the pH and ETP values then increased to 162 nM and 1780 nM min respectively. These results show that the HMVEC influence the TG assay and that incorporation into the WB assay model may well reflect *in vivo* changes to a greater extent than currently available TG assays.

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# **Whole blood thrombin and thromboelastography 'thrombus' generation assays: a comparative study in healthy control subjects and patients with a history of venous thromboembolism**

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There is considerable interest in the evaluation of thrombin generation (TG) as a risk factor for thrombosis. Thrombin generation assays have historically been performed on platelet poor and platelet rich plasmas. We have recently developed a TG assay for whole blood (WB). An alternative approach is the use of WB in thromboelastography (TEG Haemoscope Corp., USA). A development within the manufacturer's software has enabled the generated TEG traces to be manipulated to produce 'thrombus' generation curves. A comparison has been made between the two WB models by testing WB samples from 30 healthy control subjects (HCS) and 49

patients with a history of venous thrombosis (VTE). Reference ranges (5th to 95th percentile) for the TG assay (peak height [PH] 160 to 304 nM and endogenous thrombin potential [ETP] 1405 to 2323 nMmin) and the 'thrombus' generation assay (maximum rate of 'thrombus' generation [MTG] 7.0–10.9 mm × 100/second and total 'thrombus' generation [TTG] 2505 to 3146 mm × 100) were established using the WB samples from the 30 HCS. Whilst the PH and ETP values of the VTE group were significantly higher than the healthy control group values ( $P = 0.003$ ,  $P = 0.003$  respectively), there was no significant difference for the MTG or TTG values between the groups. Raised PH and/or ETP values were seen in 25 patients and raised MTG and/or TTG were seen in 17 patients. Of the 17 patients with raised MTG and/or TTG, 14 had raised PH and/or ETP. A significant correlation was observed in the VTE group between PH and MTG ( $P < 0.001$ ,  $RS = 0.46$ ) and TTG ( $P < 0.001$ ,  $RS = 0.49$ ) as well as between ETP and MTG ( $P < 0.001$ ,  $RS = 0.48$ ) and TTG ( $P < 0.0001$ ,  $RS = 0.54$ ). Both assays detected increased TG in some patients with a previous history of thrombosis.

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ABSTRACT WITHDRAWN

## **Poster Presentations: Lymphoid Malignancy**

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# **Abnormal AP-1 transcription factor protein expression in cutaneous large-cell lymphomas**

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Cutaneous large-cell lymphomas (CLCL) represent a heterogeneous subgroup of skin lymphomas including primary cutaneous CD30 + anaplastic large cell lymphoma (C-ALCL), lymphomatoid papulosis (LyP), transformed mycosis fungoides (T-MF) and Hodgkin's lymphoma (HL) with cutaneous involvement. Despite recent progress in clinical management of CLCL, the aetiology and underlying molecular pathogenesis remain elusive. The activator protein 1 (AP-1) transcription factor consists of JUN (c-JUN, JUNB and JUND), FOS (c-Fos, FosB, Fra-1 and Fra-2) and other protein families with diverse biological functions. Recent studies from our group and others have revealed up-regulation of JUNB in both MF and C-ALCL and overexpression of JUNB and CD30 in systematic HL and ALCL. To systematically assess the expression pattern of AP-1 transcription factors in CLCL, we analysed paraffin tissue sections from 27 cases of LyP, 10 C-ALCL, 8 transformed MF and 2 cutaneous HL by using immunohistochemistry (IHC) with antibodies against c-JUN, JUNB, JUND, c-FOS and RAF1. We also stained additional 7 cases of Sezary syndrome (SS), 10 C-ALCL, 6 T-MF, 3 cutaneous HL, 2 LyP and control samples with total and phosphorylated MAPK antibodies. A definitive positivity ( + + ) for JUND was seen in 13 LyP (48%), 10 C-ALCL, 6 T-MF (75%) and 2 cutaneous HL cases, and for JUNB in 4 T-MF (50%), 4 C-ALCL (44%), 3 LyP (11%) and 2

cutaneous HL. While c-JUN, c-FOS, and RAF-1 were rarely expressed in these CLCL cases. In addition, total (P44/42) Mapkinase and phosphorylated P44/42 Mapkinase were expressed in 9 C-ALCL (90%), 7 SS (88%), 5 T-MF (89%) and 3 cutaneous HL, the majority of which were also positive for JUNB. These results suggest that there is a dysregulation of AP-1 expression in CLCL mainly due to aberrant JUNB protein expression, which is up-regulated by MAPK protein, and increased JUND expression may also be relevant to CLCL.

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# **Expression of FHIT protein in hodgkin and reed-sternberg cells from chinese hodgkin lymphoma**

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Loss of the fragile histidine triad gene (FHIT) has recently been described in some human malignancies. However, little is known about the role of FHIT in the pathogenesis of Hodgkin lymphoma (HL) and the origin of HL tumour cells remains elusive. To address these issues, we investigated 33 Chinese HL cases by using B- and T-cell clonality assays and immunohistochemistry (IHC) with antibodies against FHIT, CD133/1(AC133); CD34, CD20, CD3 and c-erbB-2. IHC revealed positive staining for FHIT in 30 cases (91%). FHIT protein expression was mainly located in cytoplasm, nucleus and membrane of Hodgkin and Reed-Sternberg cells, and associated atypically spindle cells. In addition, monocytes, histiocytes and



dendritic histiocytic cells were also positive for FHIT. However, normal and reactive B and T lymphocytes and their tumour counterparts were negative for FHIT protein. T- and B-cell clonality analysis showed that 12 HL cases had TCR-r and TCR-b clones (36%), of which one case also showed IgH clone in tumour cells. All of the HL cases analysed were negative for haematopoietic cancer cell marker CD133/1(AC133), haematopoietic stem cell marker CD34, T-cell marker CD3 and oncoprotein c-erbB-2. Only 2 cases were positive for B-cell marker CD20. These results suggest aberrant FHIT protein expression may be a common feature of HL although the nature of expressed FHIT protein remain unknown, and HL tumour cells may originate from histiocytic lineage or its progenitor cells rather than lymphocyte, haematopoietic stem cells and haematopoietic cancer cells.

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# **Quantitative and qualitative assessment of constitutive nuclear factor kappa B(NF-kappa B) expression in CLL cells and its relationship with *in vitro* spontaneous apoptosis**

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NF-kappaB is part of a multi-component signaling pathway that regulates the expression of hundreds of genes involved in the control of cell proliferation, cell survival, stress responses, innate immunity and inflammation. The principal constituents of mammalian NF-kappaB are p65, p50, p52, c-Rel and Rel-B and in their inactive state they are bound in the cytoplasm to the inhibitor protein IKB. Phosphorylation of IKB by IKB kinase leads to the translocation of NF-kappaB into the nucleus where it can bind to kappaB responsive elements.

It has been reported that NF-kappaB is constitutively activated in chronic lymphocytic leukaemia (CLL) cells. However, no previous studies have prospectively quantified the sub units of NF-kappaB in CLL cells or examined their relative expression between patient samples. Therefore, the aim of our study was to quantify the expression of NF-kappaB in freshly isolated CLL cells and examine their relationship with *in vitro* viability. NF-kappaB was evaluated using EMSA, supershift assays and subunit-specific ELISA ( $n = 50$ ). Apoptosis was measured at 24h and 48h using annexin V/propidium iodide labeling. We demonstrated p50, p65 and C-rel were the predominant NF-kappaB subunits in all the CLL samples studied. Importantly, we showed a negative correlation between *in vitro* apoptosis and nuclear p65 NF-kappaB expression ( $r^2 = -0.34$ ) and a positive correlation ( $r^2 = 0.65$ ) with absolute lymphocyte count (ALC). This suggests that high levels of p65 NF-kappaB are cytoprotective. Furthermore, the positive correlation with ALC points to a role for p65 in disease progression.

This study is now being extended to a larger number of patient samples in order to establish whether p65 expression can be used to accurately stratify patients according to risk of disease progression and to investigate the correlation of NF-kappaB level with other prognostic markers including CD38, ZAP-70, VH mutational status and clinical staging.

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# **Development of monoclonal serum paraprotein following heart or lung transplantation, with a case report of post-transplant multiple myeloma: a 7-year study from a UK tertiary centre**

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There is little data published concerning the development of serum monoclonal paraprotein following heart and lung transplantation. Its role as a risk factor for the development of subsequent post-transplant lymphoproliferative disorder is poorly understood.

We reviewed patients receiving heart, single lung and double lung transplants at a single tertiary centre, and analysed those developing significant monoclonal paraproteins.

Two hundred and thirty-six patients received organ transplants. Three developed a significant monoclonal paraprotein. One was diagnosed with multiple myeloma using criteria applicable to the non-transplant setting. This case is reported in detail.

There was discrepancy between the rate of monoclonal paraprotein production found in heart and lung transplant patients in our centre compared with previous reports from patients receiving lower intensity immunosuppressive therapy following renal transplantation. This challenges the significance of monoclonal paraprotein after organ transplantation as a risk for subsequent development of post-transplant lymphoproliferative disorder. The case reported is only the third case of Ebstein-Barr negative post-transplant multiple myeloma, and it is therefore difficult to draw significant conclusion from this result. We would encourage further collection of cases to establish whether intensity of immunosuppression following solid-organ transplantation is a risk for development of the disease, and to develop treatment strategies.

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# **ZAP 70 expression in acute lymphoblastic leukaemia could have clinical prognostical significance**

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Changes in mRNA expression of three fold may be required for differential detection by microarray analysis and this technology may limit the potential information to be derived from the study of ZAP-70 expression levels. We examined the expression of ZAP-70 in 76 adult patients with ALL (64 B lineage and 12 T lineage) using real-time quantitative PCR analysis.

RNA was extracted from diagnostic bone marrow specimens taken from 76 patients aged over 18 years presenting with pre-B ALL. A wide range of ZAP-70 expression was seen from a ratio of 0.002 to 5.3 with an average of 0.332 and median of 0.185. Expression of ZAP-70 showed a relatively continuous pattern of expression across the cohort apart from six samples (8%) with a level of ZAP-70 expression above that of the Jurkat T-cell line.

Statistical analysis between ZAP-70 expression and cytogenetic subgroup was performed using either a two-sample test or ANOVA

and correlation analysis was evaluated using the Pearson coefficient. No statistically significant association between ZAP-70 expression and any cytogenetic subgroup were found. All four cases of *t*(11;19) with E2A/PBX1 gene fusion in our cohort had ZAP-70 levels above the median. Observations of interest were (a) high expression in T-ALL subgroup of patients with 11/12 ratios above the median, and a mean of 0.26 (b) low expression in the b3a2/b2a2 p210 BCR/ABL subgroup with 4/5 patients having ratios below the median. Clinical information on the top 3 patients who expressed ZAP-70, greater than/equal to 1.3, revealed failure to achieve remission with induction chemotherapy.

We used quantitative PCR to confirm that ZAP-70 is expressed in the majority of cases of ALL and demonstrate wide range of expression between cases. Further studies are required to confirm the clinical significance of ZAP-70.

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### **A phase I/II trial of sirolimus in combination with oral cyclophosphamide (C-weekly) and dexamethasone (SCD) in patients with relapsed myeloma**

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Sirolimus (SRL, rapamycin) is a commercially available oral drug that acts as an inhibitor of mTOR, a tyrosine kinase that functions as a cellular integrator of environmental growth signals through its binding to cytoplasmic FKBP12, and of NF-kappaB through binding to FKBP51. Early results of a single agent study with temsirolimus, a prodrug of SRL, showed a response in six out of 14 relapsed/refractory myeloma patients. SRL has also been shown to sensitize various myeloid and lymphoid malignancies to conventional chemotherapy. Since the therapeutic potential of SRL may be greatest when it is given in combination with conventional chemotherapy, we set out to perform a dose finding study according to the 3 + 3 design of C-weekly at incremental doses in successive cohorts together with fortnightly 4-day pulses of dexamethasone 20 mg od and SRL 5 mg od, adjusted to achieve whole blood trough levels of 8–16 ng/ml. So far eight patients have been enrolled (age 49–77), all with Salmon-Durie stage IIIA disease, five after at least one autograft earlier in the course of their disease. The starting C-weekly dose was 200 mg. Two patients, both with significant cytopenias during earlier courses of chemotherapy, one each in the cohorts receiving 300 and 400 mg of C-weekly, had to come off study because of grade 3 thrombocytopenia or neutropenia that took longer than 3 weeks to resolve, but there was no unmanageable haematological or any significant non-haematological toxicity other than diarrhoea responsive to loperamide (two patients). All seven patients evaluable by the Bladé criteria showed a response (six partial responses, one minimal response). The study is continuing to enrol at C-weekly 400 mg. We conclude that in relapsed myeloma, SCD is an active oral chemotherapy combination with manageable haematological toxicity that warrants evaluation in a randomised controlled phase II study.

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### **Stroke-like syndrome secondary to methotrexate neurotoxicity**

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Neurological toxicity secondary to intravenous or intrathecal methotrexate (MTX) is a potentially devastating complication of therapy for haematological malignancy.

We describe three cases of methotrexate neurotoxicity in young adult patients. Two of the patients were undergoing phase II induction as per the current UKALLXII protocol and one as per the GMALL protocol. Toxicity was manifested by an acute stroke-like syndrome with characteristic onset and clinical features. The patients developed a fluctuating neurological deficit characterised by altered conscious level, expressive dysphasia, dysarthria and unilateral weakness. In each case onset was within ten days of administration of intrathecal methotrexate. Characteristic multiple bilateral deep white matter changes were observed on MRI scan in the acute stage and on follow up. All recovered spontaneously over a period of days to weeks. The two patients on the UKALLXII protocol were subsequently re-exposed to intravenous MTX without re-occurrence of symptoms.

We also describe a fourth case with relapsed ALL who suffered fatal neurotoxicity secondary to methotrexate during re-induction with the UKALL2003 protocol. The patient had previously received cranio-spinal irradiation and we speculate that this led to enhanced neurotoxicity and a fatal outcome in this case.

A high index of suspicion is required for the diagnosis of MTX-related neurotoxicity. Recognition of its occurrence is particularly important given that MTX is a major component of CNS directed therapy within current therapeutic protocols for ALL. Fortunately patients normally make a full neurological recovery and importantly can usually be retreated with MTX without incident. Patients who have received previous CNS radiotherapy are at higher risk of significant and potentially irreversible CNS toxicity. Other risk factors include previous MTX-related neurotoxicity and active CNS leukaemia. These factors should be taken into consideration when planning re-induction therapy.

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### **Impairment of peripheral blood stem cell mobilisation in patients with mantle cell lymphoma following primary treatment with fludarabine-based chemotherapy**

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Fludarabine-based chemotherapy is highly active in newly diagnosed and pre-treated mantle cell lymphoma (MCL). However, concerns remain about its potential toxicity to haematopoietic stem cells and the subsequent difficulty in mobilising sufficient numbers for transplantation. The National Cancer Research Network is currently coordinating a phase III randomised study (LY05) comparing fludarabine and cyclophosphamide (FC) ± rituximab (R) as initial therapy for previously untreated MCL. We undertook an analysis of

patients entered within this trial in whom mobilisation had been attempted to assess the frequency of success.

Thirteen patients (median age 53, range 44–61) were evaluable. Stem cell collection was attempted after a median of four cycles (range 3–6) of chemotherapy (FC,  $n = 7$ ; FCR,  $n = 6$ ). Twenty-two leucapheresis procedures were performed after a variety of mobilisation schedules. Only three patients (23%) achieved an adequate stem cell yield (greater/equal to  $2 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells). This was despite a considerable delay (up to 20 months) between fludarabine exposure and mobilisation therapy in some patients. In all three cases, successful harvest followed only one mobilisation attempt using a combination of cyclophosphamide  $1.5 \text{ g/m}^2$  ( $n = 1$ ) or  $3 \text{ g/m}^2$  ( $n = 2$ ) plus GCSF  $5 \text{ mcg/kg/day}$ . Stem cell collection was unsuccessful in the remaining 10 patients despite one mobilisation attempt in three cases, two in five cases, and three in two cases.

Our finding is in contrast to other publications reporting higher frequencies of successful mobilisation post-fludarabine therapy in indolent lymphoproliferative disorders other than MCL. There is also good evidence that stem cell mobilisation among patients with MCL is possible following non-fludarabine-containing therapy such as CHOP. On the basis of our data, we suggest that patients with MCL considered candidates for autologous stem cell transplantation should receive non-purine analogue-containing chemotherapy prior to mobilisation. If fludarabine-based therapy is preferable, early stem cell mobilisation after two cycles of treatment should be considered.

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### Angiogenesis factors pattern is different in acute lymphoblastic leukaemia and chronic lymphocytic leukaemia

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Angiogenesis is a crucial event in the survival and progression of solid tumors. The angiogenic status and the exact role of the angiogenic cytokines in lymphoid leukemia is not fully clear.

In this context, we have investigated the profile of the systemic components of angiogenic regulation in B-lineage acute lymphoblastic leukemia (B-ALL) and B-chronic lymphocytic leukemia (B-CLL) serum levels of vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-alpha), endostatin and matrix metalloproteinase-9 (MMP-9) by enzyme-linked immunosorbent assay (ELISA).

In B-ALL patients, sVEGF, and MMP-9 were significantly lower than control level at diagnosis ( $P < 0.001$ ,  $P = 0.004$ ) and increased near to the control levels in remission ( $P > 0.05$ ). Both serum TNF-alpha and endostatin levels showed no significant difference both at diagnosis ( $P > 0.05$ ) and in remission ( $P > 0.05$ ) comparing to control levels. sVEGF, sTNF-alpha, sMMP-9 and endostatin level were not significantly correlated to peripheral white cell count or bone marrow blast cells count, however, positively correlated to platelets count.

In B-CLL patients, serum VEGF, sMMP-9 and sTNF-alpha were significantly higher ( $P < 0.001$ ,  $P = 0.009$ ,  $0.007$ , respectively) and decreased near control levels in remission ( $P > 0.05$  for all). Serum endostatin levels showed no significant difference at diagnosis and in remission compared to control levels ( $P > 0.05$ ). Significant positive correlation between sVEGF, sTNF-alpha, sMMP-9 and peripheral white cell counts and bone marrow lymphocytic count, and platelets count were detected.

In conclusion our data suggest that the driving forces of angiogenic factors (VEGF, TNF-alpha, MMP-9) in a adult B-ALL appears different from that in B-CLL patients. Further investigation on the biology of angiogenesis in ALL is required.

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### Neoplastic plasma cells are demonstrable at bone marrow sites distant to solitary plasmacytoma of bone and predict for progression to multiple myeloma

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Approximately 50% of patient's with solitary plasmacytoma of bone (SPB) progress to myeloma. Identification of high-risk patients at the time of diagnosis would enable risk stratification and more careful monitoring of patients. Although adjunctive chemotherapy has not definitively been shown to benefit unselected patients with SPB, future therapeutic advances might be targeted on patients with a high risk of progression. We and others have previously demonstrated that neoplastic bone marrow plasma cells are distinguishable from their normal counterparts by virtue of their lack of CD19 expression and/or their aberrant expression of CD56. We developed a multiparameter flow cytometry assay, which predicts outcome following autologous transplantation in myeloma patients and risk of progression in patients with MGUS. We applied this assay to staging bone marrow specimens from patients with biopsy proven SPB for the presence of occult disease at sites distant to the primary lesion. Fifty-two patients were included (31 male, 21 female, median age 65-year) and in each case the staging bone marrow was not indicative of myeloma ( $< 10\%$  plasma cells). Plasma cells comprised a median of  $0.6\%$  ( $0.05\text{--}6.2\%$ ) of bone marrow leucocytes while distinct populations with a neoplastic immunophenotype ( $> 30\%$  CD19– and/or CD56+ as per convention) were demonstrable 35/52 (67%). Neoplastic plasma cells when present comprised a median of  $70\%$  (35–100%) of bone marrow plasma cells. Twenty-one patients (40%) developed myeloma with a median time to progression of 476 days (range 18–1632). Progression occurred in 18 of the 35 (51%) patients with neoplastic plasma cells in their staging marrows and in 3/17 (18%) patients with a normal phenotypic profile. The difference was significant using Chi-square analysis with Yates' correction for continuity ( $P = 0.04$ ). We would conclude that neoplastic plasma cells are frequently found at bone marrow sites distant to SPB and that their presence predicts for progression to multiple myeloma. Trials of adjuvant systemic therapy are warranted in this group.

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### Cytogenetic classification of T lineage acute lymphoblastic leukaemia

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Increasing numbers of genetic changes are being described in T lineage acute lymphoblastic leukaemia (T ALL), which may be used to classify patients into subgroups and define multi-step oncogenic pathways. We have integrated the significant abnormalities into a comprehensive genetic classification of T ALL, using appropriate probes for fluorescence *in situ* hybridization (FISH). This approach revealed new recurrent translocation partners, as well as determining the incidence and simultaneous occurrence of the different abnormalities. The series included 295 patients, children 0–14 years ( $n = 206$ ) and adults of 15 years and above ( $n = 89$ ), with a diagnosis of T ALL, entered to one of the UK MRC/NCRI ALL treatment trials. The incidences of the common cryptic abnormalities, *SIL-TAL1* fusion and *TLX3* were more prevalent in children (20% and 17%,

respectively) compared to adults (9% each). There was no difference in event free survival between the childhood patients with *SIL-TAL1* fusion and *TLX3* rearrangements. *CALM-AF10* fusion and *MLL* rearrangements accounted for 4% each. A single patient was found with a *BCR-ABL* fusion, but the same probe identified nine (3%) with *NUP214-ABL1* amplification. Deletions involving *CDKN2A* were present in 49% of patients, in association with all abnormalities. Concurrent rearrangements were found between the *TCR* genes, as well as associations between *MYC*, *IGH* and the other oncogenes. FISH detected several novel, recurrent rearrangements. These findings demonstrate the valuable role of FISH analysis, with a panel of carefully selected probes, to classify T ALL patients into genetic subgroups, including rare variants, and provide information on the relationship between them. A metaphase FISH approach has facilitated the identification of potential new target genes. In particular, multiple partners of *TRB@* and *BCL11B* have emerged, highlighting the importance of these genes in the pathogenesis of T ALL.

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# **Toxicity of fludarabine and cyclophosphamide (FC) ± rituximab (R) as initial therapy for patients with previously-untreated mantle cell lymphoma: results of a randomised phase II study**

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**Background:** The combination of fludarabine and cyclophosphamide is well recognized as being significantly immunosuppressive and there are concerns that the addition of rituximab to this regimen may increase the frequency of infectious complications. The impact of rituximab on other markers of toxicity is also unclear.

**Methods:** The National Cancer Research Network (NCRN) is currently coordinating a phase III randomized study (LY05) comparing fludarabine and cyclophosphamide (FC) ± rituximab<sup>®</sup> as initial therapy for patients with previously-untreated mantle cell lymphoma. Prior to the phase III study, the same design phase II study was conducted. The outcome measures for the phase II study are response and toxicity.

All toxicity was graded according to the National Cancer Institute toxicity criteria grading scale.

**Results:** A total of 139 patients were randomised in the phase II study. Non-haematological toxicity was similar between the two treatment arms. The only significant difference in haematological toxicity was a higher rate of leucopenia with FCR chemotherapy compared to FC chemotherapy (FCR 58.0% vs FC 41.4%,  $P = 0.0244$ ). This was due to lymphopenia rather than neutropenia since the frequency and severity of the latter was unaffected by the addition of rituximab (FC 50.0% vs FCR 60.9%,  $P = 0.2213$ ). Most importantly, the higher rates of leucopenia with FCR did not translate into a significantly increased number of febrile episodes (FC 1.6% vs FCR 9.0%,  $P = 0.5483$ ) or infections (FC 9.7% vs FCR 20.0%,  $P = 0.2406$ ). All percentages quoted relate to grade 3 or 4 toxicity only. Conclusions the addition of rituximab to fludarabine and cyclophosphamide as initial treatment for previously untreated mantle cell lymphoma has no significant impact on toxicity. The higher rates of leucopenia produced by FCR chemotherapy are due to lymphopenia rather than neutropenia and do not result in more frequent febrile episodes or infections.

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# **CC-5013 (revlimid, lenalinomide) induces *in vitro* PBMC-toxicity against mantle cell lymphoma and this is enhanced by rituximab**

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Mantle cell lymphoma (MCL) is an incurable condition in most patients and with a median presentation in the mid-60s new treatment modalities are clearly needed. Revlimid, a second generation immunomodulatory drug (IMiD), which is based on thalidomide has shown promising results in multiple myeloma and chronic lymphocytic leukaemia, with a more favourable toxicity profile.

This study aims to examine *in vitro* both the direct effects of Revlimid on patient-derived MCL cells and cell lines (MCL cell viability) and the indirect effects of Revlimid on the immune system (PBMC-mediated toxicity, complement-dependent toxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).

Revlimid had no significant direct effect on the viability of MCL cell lines (Granta 519 and SP53), or on patient-derived MCL cells. However, in one patient with blast cells, a specific reduction in blast cells was observed. Rituximab alone and in combination with Revlimid did not adversely affect viability.

Incubation of Revlimid with both patient derived or healthy control PBMCs significantly enhanced their toxicity against Granta 519 cells when compared with DMSO control-treated cells ( $P < 0.01$ ). CC-5013 was also capable of enhancing the toxicity of anti-CD3-stimulated T cells against Granta 519 when compared to controls ( $P < 0.01$ ). Pre-treatment of MCL cells with Revlimid did not alter their susceptibility to CDC, both in the presence or absence of Rituximab. Co-culture of Revlimid pre-treated PBMCs with rituximab-treated MCL cells resulted in enhanced ADCC when compared with DMSO pre-treated PBMCs.

We conclude that Revlimid does not appear to have a significant direct effect on MCL cell viability or on CDC, but that it is capable of enhancing PBMC-mediated toxicity both in the presence and absence of Rituximab.

A NCRN clinical trial using Revlimid in MCL is planned to start later this year.

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# **Tropical splenic lymphoma: detailed clinico-pathological assessment in 19 Ghanaian patients**

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We have previously described a subgroup of West African patients with massive splenomegaly who appear to have a clinically distinctive lymphoproliferative disorder (LPD) termed tropical splenic lymphoma (TSL). These patients tend to be female, relatively young and an aetiological link with malaria was postulated. Only limited phenotypic and genotypic characterization was previously possible. We have therefore evaluated the clinical and laboratory features of 19 patients with a clinical diagnosis of TSL to determine whether TSL represents a distinct entity or should be considered as splenic marginal zone lymphoma (SMZL). Peripheral blood and bone

marrow aspirate and trephine biopsies were assessed at the HMDS laboratory, Leeds. The median age was 63 years, 73% were female and the median spleen length was 17 cm. Five out of nineteen patients had lymphadenopathy and seventeen out of nineteen patients had a lymphocytosis (median  $30.1 \times 10^9/l$ ). Marrow infiltration was evident in all patients and was frequently extensive. The majority of cases were characterised by a CD5<sup>+</sup> CD10<sup>+</sup> CD20<sup>+</sup> CD23<sup>+</sup> CD79<sup>+</sup> BCL2<sup>+</sup> BCL6<sup>+</sup> MUM1/IRF4<sup>+</sup> cyclin D1<sup>+</sup> immunophenotype. The rate of cell proliferation was low in all cases. FISH studies demonstrated del7q31 in four out of eighteen cases and del6q21 in one out of nineteen cases but there was no evidence of the t(11;14), t(9;14) or MALT1 rearrangements. IgH sequence analysis was performed in 16 cases and demonstrated that nine out of sixteen cases were germline and seven out of sixteen were mutated. Within the mutated group the overall mutation load appeared to be relatively low median 3.3% with most utilising VH3 family genes. This is the first detailed clinicopathological assessment of TSL. These patients have some pathological features seen in patients with SMZL such as CD5<sup>+</sup> CD10<sup>+</sup> CD23<sup>+</sup> immunophenotype, 7q31 deletions and cases with both germline and mutated Ig genes. Definitive phenotypic and genotypic features are lacking in SMZL and it remains uncertain whether TSL represents a distinct clinicopathological entity.

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### Protein kinase C betaII is overexpressed in B-cell lymphocytic leukaemia

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B-cell chronic lymphocytic leukaemia (CLL) is an incurable disease of mature B lymphocytes that becomes malignant in response to uncharacterised oncogenic events. This response may involve constitutive signalling within malignant cells resulting in escape from apoptosis. One such signal could involve constitutively active Protein kinase C (PKC). PKC is a serine-threonine kinase involved in an array of biological processes ranging from cellular proliferation to differentiation and cell survival. Mammalian PKC consists of 12 different isoforms.

Little was known about the relative expression of these isoforms in CLL cells. The aim of this study was to establish a definite and quantitative expression pattern of PKC isoforms in CLL cells. The expression pattern was compared to that of normal B cells and hairy-cell leukaemia cells. In CLL, PKCs have been identified as therapeutic targets and PKC modulating agents are already under clinical trials. It is believed that knowledge of PKC expression pattern would aid in development of more targeted therapies for CLL patients.

The method involved separation of cellular proteins from CLL, HCL and normal B cells using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and developing Western blots using anti-PKC antibodies. Purified recombinant PKC proteins were used as standards. Following densitometry PKC concentrations within the cell lysates were calculated.

In the present study I established the profile of PKC isoforms in CLL cells showing that CLL cells express PKC alpha, betaI, betaII, delta, epsilon, mu, zeta and iota. I also showed, for the first time that among all isoforms, PKC betaII is over-expressed in these cells.

Further to this it has been shown that overexpression of PKC betaII is a unique feature of CLL cells and the activity of this enzyme strongly correlates with CLL-cell response to B-cell receptor engagement (Abrams ST and Lakum T *et al.* [2006] *Blood* Sept 26 Epub ahead of print).

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### Profiling of proteasome catalytic activities in chronic lymphocytic leukaemia

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Chronic lymphocytic leukaemia (CLL) is the commonest adult lymphoid malignancy characterized by peripheral blood lymphocytosis and affecting predominantly the older patients. The proteasome is a multi-catalytic protease responsible for regulating intracellular protein degradation. Proteasome inhibition is an emerging novel therapy in haematological malignancies and bortezomib has already shown efficacy in the treatment of multiple myeloma. Proteasome function is mediated by three main enzyme activities, namely, chymotrypsin-like (CT-L), trypsin-like (T-L) and peptidylglutamyl peptide hydrolysing (PGPH) activities.

Fluorogenic assays were used to investigate the level of the three activities of the proteasome. Proteasome activity was measured by monitoring the release of the fluorophore aminomethylcoumarin (AMC) from peptide substrates specific for each activity (Succ-LLVY-AMC for CT-L, Z-ARR-AMC for T-L and Z-LLE-AMC for PGPH). Peripheral blood was collected in an EDTA bottle from newly diagnosed and known CLL patients. Mononuclear cells were separated using Ficoll and proteasomes extracted using an ATP/DTT lysis buffer. Lysates were stored at  $-80^{\circ}\text{C}$  and batched for enzyme assay. Results are expressed in arbitrary fluorescence units (AFU) per 50  $\mu\text{g}$ .

In this group of CLL patients ( $n = 7$ , male = 4, female = 3), the mean CT-L activity was lowest (7.68 AFU, range 2.04–9.08), while the mean T-L activity was highest (58.58 AFU, range 19.09–169.94). PGPH activity had an intermediate mean value (36.68 AFU, range 12.24–69.25). In all cases tested, T-L activity was the major contributor to proteasome function. The proteasome inhibitor bortezomib has been designed to specifically target the CT-L activity. It has recently been reported that the IC<sub>50</sub> for CLL cells *in vitro* is double that for myeloma cells and that CLL patients have not responded to bortezomib *in vivo*. This preliminary study suggests that lack of response *in vivo* may reflect the low CT-L activity detected *in vitro*. Second generation proteasome inhibitors are currently being developed to target the T-L and PGPH; these may show greater efficacy in CLL.

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### Rapid infusion rituximab is as clinically effective and safe as conventional infusion regimes in the treatment of diffuse large B-cell lymphoma: a 2-year prospective study

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Rituximab is increasingly used in the treatment of CD20 positive B cell malignancies and other haematological disorders. This has placed increasing pressure on the capacity of chemotherapy units, especially given the recommended long infusion times (3–4 hours) for rituximab. Several studies have suggested that rituximab may be safely administered by more rapid infusion (90 mins) but none have examined whether this change of protocol impacts on the efficacy of the agent.

We prospectively studied 61 patients with diffuse large B-cell lymphoma (DLBCL), treated with R-CHOP chemotherapy using a rapid rituximab infusion protocol from the second treatment onwards if the initial rituximab infusion administered at the standard recommended rate, was well tolerated (NCI toxicity grade 0 or 1). Premedication of chlorphenamine 8 mg, prednisolone 100 mg, paracetamol 1 g and tropisetron 5 mg was administered 1 hour before receiving rituximab.

Of the 61 patients, 60 had no reaction with their first standard-rate infusion, only one patient had a Grade 1 reaction, meaning all could progress to our rapid infusion protocol for their remaining treatments. All rapid infusions were well tolerated with no infusion-related toxicity.

Our study demonstrated that rapid rituximab infusions are both safe and as clinically effective as those using standard infusion rates. After follow-up ranging from 7 to 25 months, complete responses were seen in 69% (42/61) of patients and overall survival was 77%. This is comparable to the GELA trial (Coiffier et al, 2002) where a complete response rate of 75% and overall survival of 70% after 2-years was observed in patients with DLBCL-treated R-CHOP by standard infusion.

We believe that rapid infusion of rituximab for DLBCL is effective and safe and should be the standard of care for patients with DLBCL to allow increased capacity of chemotherapy units and greater patient satisfaction.

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#### EBV related transformation events in CLL

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Less than 5% of patients with CLL undergo transformation to diffuse large B-cell lymphoma. Transformation to Classical Hodgkins lymphoma (CHL) is also recognised. Such events may reflect genetic changes in the CLL clone but there is evidence to suggest that at least some transformation events may be Epstein-Barr virus (EBV) related neoplasms. We have reviewed the clinical and laboratory features of 15 CLL patients with biopsy proven transformation to DLBL and two patients who developed CHL. Sections were assessed for the expression of a range of immunophenotypic markers and EBV latent membrane protein 1 (LMP-1). Of the 15 patients developing DLBL five appeared phenotypically related to the underlying CLL clone. In the remaining patients the tumour cells appeared phenotypically distinct as they lacked CD5 and CD23 and expressed germinal centre markers in some instances. LMP1 positivity was demonstrable in five patients with DLBL (one apparently related and four unrelated to the underlying CLL). Of the two patients who developed CHL one was associated with EBV and lacked CD20 expression. All the patients with EBV + tumours were heavily pre-treated (median prior therapies 4). The median time from original diagnosis to histological transformation was 74.5 months. Four patients presented with nodal disease and two patients presented with extranodal disease. The outcome of the EBV associated tumors in these patients was death in two patients, remission with intensive combination chemotherapy in two patients and spontaneous remission of nodal disease in one patient. One patient is undergoing intensive chemotherapy. We conclude that not all transformation events in CLL occur within the original clone. The majority appear to be clonally distinct and a significant proportion of these appear to be EBV associated. This presumably occurs as a result of both the underlying immune-deficiency seen in CLL as well the potent immunosuppressive agents given as therapy. At least a minority may resolve spontaneously. All biopsies demonstrating histological transformation in CLL patients should be assessed for the presence of EBV.

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#### Dysplastic haematopoiesis in untreated chronic lymphocytic leukaemia (CLL)

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In the MRC CLL-5 pilot study, 8% of patients developed myelodysplasia/acute myeloid leukaemia (MDS/AML) after treatment. It was not established whether this was an effect of treatment or due to unmasking of a concomitant dysplastic clone. We therefore assessed bone marrow aspirates of 28 untreated CLL patients for the presence of co-existing dysplasia by morphological criteria, evaluating 100 erythroid cells, 100 myeloid cells and 20 megakaryocytes in each case. Cytogenetic analysis and trephine biopsy reports (extent and pattern of lymphoid infiltration) were also reviewed. Abnormalities seen were compared with reported patterns of abnormal morphological findings in normal volunteer marrows (Bain [1996] *British Journal of Haematology*, 94, 206).

Abnormal erythropoiesis (>50% cells) was found in 26 (93%) of cases, abnormal myelopoiesis in 5 (18%) and abnormal megakaryopoiesis in 4 (14%). Erythroid abnormalities included irregular/poorly haemoglobinised/vacuolated cytoplasm (57.8% vs 7.0%,  $P < 0.0001$ ), cytoplasmic bridging (10.8% vs 6%,  $P = 0.0498$ ), nuclear irregularity/fragmentation/lobulation showing a trend to significance (3.8% vs 2.0%,  $P = 0.069$ ). Binuclearity was not increased and ring sideroblasts were not seen. Megakaryocyte abnormalities included separated nuclei (1-7 cells, in 20 cases), hypolobated/mononuclear forms (1-5 cells, 14 cases), and occasional micromegakaryocytes. Myeloid abnormalities included pseudo-Pelger cells (18 cases), hypogranularity (15), giant metamyelocytes (10), and ring-shaped nuclei (7). Cytogenetic analysis (conventional and/or FISH) in 17 of 28 patients showed 10 abnormal karyotypes (11q-, 13q-, trisomy 12, t(14;18), 17p-, X-, complex). None were diagnostic of MDS. There was no significant difference in abnormalities seen in those with normal vs abnormal cytogenetics, high vs low percentage of trephine infiltration, or diffuse vs non-diffuse marrow infiltration.

We conclude that residual haematopoiesis in untreated CLL shows significant morphological changes in all lineages, especially erythroid, as compared to normals. Cytogenetic abnormalities were not classical of MDS/AML. More sensitive methods than morphological analysis alone are needed to address whether occurrence of MDS/AML in CLL is a chemotherapy effect or due to unmasking of pre-existing MDS.

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#### CD38 expression in B-CLL is dynamic and influenced by cell contact in the leukaemic microenvironment

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In order to determine whether microenvironment derived signals might influence CD38 expression in B-cell chronic lymphocytic leukaemia (B-CLL), we compared levels of this molecule in peripheral blood (PB), bone marrow (BM), splenic red pulp (RP) and splenic white pulp (WP). In 35 paired BM and PB samples, significantly higher CD38 expression was observed on BM B-CLL cells compared to PB (27% vs 19%,  $P = 0.009$ ). Similarly, imaging of

the splenic tissue by confocal immunofluorescence microscopy revealed significantly higher CD38 expression by tumour in the WP compared to RP ( $P < 0.0001$ ), an area also infiltrated with T-lymphocytes. Cell contact derived signals within the microenvironment might thus be responsible for the level of CD38 expression in B-CLL.

To test this theory, we examined the effect of contact with autologous CD3/CD28 activated T lymphocytes using an *in vitro* system aimed at mimicking the tumour microenvironment. CD38 expression by B-CLL cells increased significantly in 15/15 cases over the 6-day culture period and was dependent on cell contact. Proliferation of tumour cells was also induced and was more marked in cases with higher initial levels of CD38. A parallel reduction in apoptosis was also observed. Immunofluorescence microscopy of B-CLL lymph node and spleen showed that levels of CD38 expression were higher in areas containing Ki67 + tumour and T lymphocytes. CD38 + B-CLL cells were frequently found in areas containing vascular endothelial cells expressing CD31, a known ligand for CD38.

These results show that CD38 expression in B-CLL is dynamic and influenced by contact with activated T lymphocytes. Expression of this molecule in the peripheral blood may thus serve as a surrogate marker for the extent of survival and proliferation signals provided by non-malignant cells in the leukaemic microenvironment.

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# **B-CLL cells secrete factors including IL-6 which inhibit T cell proliferation and activation and promote Th2 polarisation**

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Immune dysfunction, manifest as susceptibility to infection and autoimmune disease, is a major clinical feature of B-cell chronic lymphocytic leukaemia (B-CLL). Although this is in part directly caused by the tumour load, defective T-cell function is also observed even in patients with early stage disease.

T cells in B-CLL have an acquired defect in CD40L expression which we demonstrate is reversible and, contrary to previous work which suggested it is exclusively due to contact with tumour cells, demonstrate that soluble mediator(s) found in tumour supernatant (TSN) are also involved. TSN inhibited 3rd party allogeneic mixed lymphocyte reactions (MLR), CD40L upregulation and IL-2 secretion following activation of normal T cells by phorbol ester and ionomycin. Cell cycle entry and cell division following stimulation of normal T cells by ligation of CD3 and CD28 were also reduced by TSN. High levels of IL-6 were detected in B-CLL TSN from all cases studied. Antibody neutralisation of the IL-6 in TSN demonstrated restoration of both T-cell IL-2 production and CD40L expression, whilst addition of recombinant IL-6 to normal T cells inhibited CD40L upregulation and IL-2 production in a similar fashion to TSN. When normal T cells were activated in either TSN or IL-6 at similar concentrations to that found in TSN, Th2 polarisation was induced as evidenced by a 10 fold increase in the production of IL-4.

In summary, B-CLL cells secrete factor(s) including IL-6, a known adverse serum biomarker, which inhibit T-cell activation and proliferation and promote Th2 polarisation. This may in part explain the susceptibility of B-CLL patients to infection and autoimmunity and might also promote disease progression through the effects of Th2 cytokines on the survival of tumour cells. Anti IL-6 therapies are available and might improve T-cell function, abrogate the susceptibility to autoimmunity and inhibit disease progression.

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# **Management of Hodgkin lymphoma in the 'real world' – analysis of a population based cohort**

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Most data on outcomes in Hodgkin lymphoma (HL) come from results of clinical trials of therapy in selected groups of patients.

We present data on all patients diagnosed with HL at this hospital, serving a population of 400 000 in Coventry and Rugby, between January 2000 and December 2006. Patients referred for treatment from elsewhere were excluded. There were 57 patients (33 male, 24 female) – an incidence of 2 per 100 000 per year. Median age at diagnosis was 36 years (range 14–86). Twenty-four patients had Stage IA/IIA disease; two were HIV positive.

Fifty-five patients have been treated with curative intent. One patient died before starting treatment. One received only one course of ABVD before she opted for alternative therapies (alive in partial response 19 months later). However, 11 patients received attenuated therapy due to significant comorbidity (3 – cardiomyopathy [1], multiple sclerosis [1], other active malignancy [1]) or age [8]. Five patients have not yet completed treatment.

Six patients have relapsed; three have progressed during treatment (all three died). Eight patients have received salvage chemotherapy, and six an autologous stem cell transplant. One patient (relapsed post autograft) also underwent a reduced intensity allograft. Six patients are alive following salvage chemotherapy (five post autograft), 28–66 months later. There have been nine deaths, one prior to treatment, four treatment related, three due to progressive HL. There was one sudden cardiac death in a 51-year-old male smoker two years after ABVD and Mantle radiotherapy.

In our cohort, relapse beyond two years is uncommon. Probability of survival at five years is 0.81, and of disease free survival is 0.78. These data show early outcomes in an unselected population of HL patients treated in the 'real world'. We intend to observe this cohort as it matures in order to demonstrate the effects of late toxicities of therapy on survival.

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# **The t(9;14)(p13;q32) is a rare abnormality in B cell lymphoma**

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The t(9;14)(p13;q32) which deregulates PAX5 as a consequence of its juxtaposition to the IGH locus was originally described in patients with lymphoplasmacytic lymphoma. Subsequent studies have failed to confirm this finding and have demonstrated the translocation in some patients with splenic marginal zone lymphoma, diffuse large B-cell lymphoma and post transplant lymphoproliferative disorders. In order to further clarify this we have evaluated (by interphase FISH) 280 cases of B-cell lymphoma for the t(9;14). Cases of follicular lymphoma and mantle cell lymphoma were specifically excluded as they contain disease defining IGH translocations. Samples were initially screened for IGH rearrangements using a dual colour IGH breakapart probe set (Vysis 32–191 019) and cases with a split signal (indicative of an IGH rearrangement) were further evaluated for rearrangements of PAX5 using another dual colour breakapart probe set (Dako Y5413). Ninety-six cases of diffuse large B-cell lymphoma and 45 cases of extranodal marginal zone lymphoma were evaluated with this strategy but none harbored a t(9;14). A further 139 cases with CD5– lymphoproliferative disorders were also evaluated and a

single patient was found to have a  $t(9;14)$ . This patient had the clinical and laboratory features typical of splenic marginal zone lymphoma.

We would therefore conclude that the  $t(9;14)$  is a very rare abnormality in B-cell lymphoma but it may be seen in a minority of patients with splenic marginal zone lymphoma.

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### Effect of cloretazine on acute myeloid leukaemia blasts *in vitro* – as a single agent and combined with cytarabine and daunorubicin

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Current chemotherapy strategies induce remission in the majority of AML patients; however up to 70% subsequently relapse, highlighting the need for new therapies. Cloretazine (VNP40101M) is a novel alkylating agent (Vion Pharmaceuticals Inc., New Haven, CT, USA). On administration, Cloretazine undergoes activation to form 90CE, a DNA chloroethylating species which chloroethylates the O6 position in guanine residues, resulting in inter-strand DNA cross linkage, cytotoxicity and ultimately cell death.

We have investigated the effect of Cloretazine on cell proliferation, viability and apoptosis of AML blasts *in vitro*, both alone and in combination with other chemotherapy agents (Cytarabine/Daunorubicin).

Blast cells were isolated by density centrifugation from 10 patients at presentation (BM or PB). Cells were cultured in 96-well plates at  $1 \times 10^6$  cells/ml in McCoy's 5A medium supplemented with 15% FCS, GM-CSF (100 ng/ml), SCF and IL-3 (10 ng/ml). Cloretazine (0, 1, 5, 10 or 20 µg/ml) was added alone or in combination with Cytarabine/Daunorubicin (10 100 or 500 ng/ml) at establishment of cultures. Cultures were incubated at 37°C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> for 72–96 h before cell proliferation, viability and apoptosis were measured using tritiated thymidine uptake, WST-1 and Annexin V staining methods, respectively.

All patients studied showed a dose dependent response to Cloretazine alone. Combination with Cytarabine or Daunorubicin at a range of concentrations showed increased effects e.g inhibition of proliferation 50% (24–92) (mean (range) with Cloretazine (5 µg/ml) alone), 71% (32–98) Cytarabine(100 ng/ml) alone increasing to 86% (58–100) when combined, 57% (20–89) Daunorubicin(10 ng/ml) alone increasing to 73% (28–96) combined. Similarly increases in cytotoxicity were observed, 28% (0–50) (Cloretazine alone), 43% (0–86) Cytarabine alone to 52% (15–92) in combination. 22% (1–70) Daunorubicin alone to 36% (0–78) combined. Apoptosis levels also showed increases with combinations.

This pilot study indicates Cloretazine is effective on AML blasts *in vitro* both alone and in combination with other chemotherapy agents. Cloretazine is currently undergoing clinical trials.

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### Gene expression profiling can identify novel MRD markers for AML

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The monitoring of minimal residual disease (MRD) has become an important issue for the diagnosis and clinical management of

patients with acute myeloid leukaemia (AML). The majority of patients <60 years of age will enter remission but at least 50% will subsequently relapse. For those patients presenting with either  $t(8;21)$ ,  $t(15;17)$  or an  $inv(16)$  (abnormalities associated with a favourable risk group) molecular markers have already been established (AML1-ETO, PML-RARα and CBFβ-MYH11) but are limited to detection by RT-PCR. As 'Proof of Principle', gene expression data from 350 patients identified several genes that were specifically, and uniquely, over-expressed for each of the three favourable risk sub-groups including genes related to the genetic abnormalities. Quantitative RT-PCR was used to monitor changes in expression of some of these genes and this was correlated with monitoring levels using established MRD markers and the clinical course of the patients. The new MRD markers tracked with the existing markers.

Unfortunately, the majority of patients, including those with a normal karyotype (NK), have no leukaemia-specific markers for MRD monitoring, although a few genes are mutated that have a noticeable effect upon a patient's outcome. NPM1 (Nucleophosmin) mutations occur in about 50% of NK patients and are generally associated with a good prognosis. The presence of FLT3 (Fms-like tyrosine kinase 3) mutations is seen as a poorer indicator, even when present alongside an NPM1 mutation. In the cohort of AML patients with NK ( $n = 121$ ) with expression profiles, a similar approach was taken to identify several specific and unique expressed genes in patients with a normal karyotype and an NPM1 mutation compared to those without. These included homeobox proteins, which were over-expressed by three fold in the NPM1 mutated group. These genes are being assessed in a series of patients to confirm suitability for MRD monitoring.

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### Mutations of the gene encoding SHIP1 are rare and SHIP1 expression levels unaltered in caucasian acute myeloid leukaemia patients

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SHIP1 (SH2-domain-containing inositol 5'-phosphatase 1) is predominantly expressed in haematopoietic cells, and negatively regulates PI3K-initiated cell signalling. SHIP1<sup>-/-</sup> knockout mice show increased haematopoietic stem cell numbers and proliferation, suggesting that SHIP1 acts as a tumour suppressor in haematopoietic cells. Consistent with this, a recent study identified mutations in the gene encoding SHIP1 in 22% of Chinese AML patients (Luo *et al.* [2004] *Journal of Experimental Hematology* 12, 420). We aimed to determine the incidence of SHIP1 mutations and mRNA levels in Caucasian AML. Group 1: Genomic DNA from 57 adult *de novo* AML cases representing all FAB subtypes was studied. PCR amplicons corresponding to all 27 exons of SHIP1 were analysed for sequence alterations by DHPLC and potential changes confirmed by DNA sequencing. Group 2: SHIP1 mRNA expression profile was



determined in a separate group of 285 AML plus five controls. RNA from blasts and mononuclear cells was analysed using Affymetrix U133A GeneChips. Group 1: two missense (one novel; c.466G>A, R156Q in 1/57 patients (2%), one polymorphic), six synonymous (four novel) and 34 untranslated sequence alterations (20 novel) were identified. No changes identified correspond to those present in a cohort of 32 Chinese AML patients (seven different missense mutations in seven patients) (Luo *et al.* [2004] *Journal of Experimental Hematology* 12, 420). Furthermore, most sequence changes identified in our study are polymorphic and therefore unlikely to elicit any phenotypic effect. Group 2: Compared to normal subjects, SHIP1 expression level was unaltered in the AML group overall and following stratification into FAB subtypes or into 16 clusters defined by expression profile. We conclude that SHIP1 mutations and altered expression do not appear to play an important role in the pathogenesis of AML in the Caucasian population in contrast to the Chinese study (Luo *et al.* [2004] *Journal of Experimental Hematology* 12, 420). These findings suggest that therapeutic trials should consider possible ethnic/geographical differences. However, further investigations are required to exclude pathogenic SHIP1 post-translational modifications in AML.

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### Heterogeneous prognostic impact of derivative chromosome 9 deletions in chronic myelogenous leukaemia

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Der(9) deletions are seen in 10–15% of CML patients and have been associated with a poor prognosis, however no studies have been performed in the context of a randomized clinical trial. We developed a DNA-based deletion screen and investigated 339 chronic phase patients treated with interferon- $\alpha$  (IFN) as first line therapy in three controlled German studies with a median observation time of 7 years. Deletions were detected in pretreatment DNA of 59/339 (17%) patients. Of these, 21 spanned the *ABL/BCR* junction and 38 were centromeric ( $n = 20$ ) or telomeric ( $n = 18$ ) of the breakpoint. There was no significant difference in overall survival between deleted and non-deleted patients. Patients with breakpoint-spanning deletions had poorer survival compared to patients without deletions (4.7 vs 7.8 years;  $P = 0.003$ ) but this was not significant when censored at allogeneic stem cell transplantation ( $n = 129$ ) or imatinib ( $n = 62$ ) treatment in first chronic phase ( $P = 0.08$ ). Unexpectedly, deletions that did not span the breakpoint were associated with improved survival compared to cases without deletions ( $P = 0.001$ ). Multiple Cox regression analysis indicated that deletion status ( $P = 0.007$ ), age ( $P = 0.018$ ) and spleen size ( $P < 0.001$ ) were significant independent indicators of survival and confirmed that only deletions spanning the *ABL/BCR* breakpoint are associated with an adverse prognosis ( $P = 0.039$ ).

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### Bone marrow trephine findings in CMML

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**Objective:** To identify characteristic features in bone marrow trephines of CMML (Chronic Myelomonocytic Leukaemia).

**Patients and methods:** A retrospective analysis of the bone marrow trephine features was performed in 22 patients. All patients had a sustained raise in peripheral blood monocyte count of over  $1 \times 10^9/l$ . **Results:** The average age of the patients is 67.7 year. The mean peripheral blood monocyte count was  $5.84 \times 10^9/l$ . All the CMML patients had hypercellular marrow with an abnormally high M:E ratio (in excess of 4 in most cases). Fifteen of the cases had noticeably increased numbers of monocytes on H&E staining, while in the other seven cases the monocytes were identified by CD68 (PGM-1) staining. The CD34 count was marginally elevated. CMML2 was diagnosed in two cases where the CD34 count was 5% and 10%. The number of megakaryocytes were increased in 11 cases and dysmegakaryopoiesis was present in 14 cases. Most (20 cases) had increased reticulin.

**Conclusion:** Appreciation of hypercellularity, high M:E ratio, increased proportion of monocytic cells (either on morphology or aided by CD68 [PGM-1]) and presence of dysmegakaryopoiesis can aid in the diagnosis of CMML in bone marrow trephine sections.

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### Bone marrow trephine findings in AML with multi-lineage dysplasia

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**Objective:** To identify characteristic features in bone marrow trephines of acute myeloid leukaemia (AML) with multi-lineage dysplasia.

**Patients and methods:** A retrospective analysis of the bone marrow trephine features was performed in 14 patients. The cases were subdivided into two groups: (a) AML with background multilineage dysplasia (AML-MD) (11), (b) Myelodysplastic syndrome which subsequently transformed to AML (MD-AML) (3).

**Results:** Nine out of eleven AML-MD patients had hypercellular marrow and increased proportion of precursor cells could be identified on morphology and CD34/HLADR/CD117 immunostains. Four cases showed trilineage dysplasia and five cases showed bilineage dysplasia. Most had increased reticulin. Megakaryocytes were increased in number in seven cases and were dysplastic in all cases but one (marked in seven). The three MD-AML cases show frank features of AML with increased numbers of precursor cells with appropriate immunophenotype and all of them showed persistent dysmegakaryopoiesis, and increased numbers of megakaryocytes, apoptoses and reticulin.

**Conclusion:** Assessment of an increased proportion of immature myeloid cells by their morphology and immunophenotype and appreciation of dysplastic features in the haemopoietic lineages based on morphological features can aid in the diagnosis of AML with multilineage dysplasia in bone marrow trephine sections.

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### Successful treatment of myelodysplasia-associated Pyoderma Gangrenosum with topical tacrolimus

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We report the case of a 71-year old with refractory anaemia with ringed sideroblasts diagnosed in November 2005 with normal cytogenetics on a FISH panel (INT-1). As a result of 2–3 weekly

blood transfusions, his ferritin rose to above 1400 in September 2006. He was started on dextranferrioxamine (DF) subcutaneous infusion. The first needle was left in-situ for 5 days rather than being replaced every 72 hours as instructed. Shortly after this, he noticed erythema and induration at the infusion site and over the next few days, this area ulcerated with purulent discharge. Swabs grew *Staphylococcus aureus*, but the ulceration continued to spread despite adequate antibiotic therapy to a 20 × 15 mm ulcer in his right iliac fossa with bluish, undermined margins and copious purulent discharge. The features were classical for pyoderma gangrenosum (PG). He was commenced on topical tacrolimus 0.1% ointment once daily under Mepilex border dressing along with oral flucloxacillin. The ulcer healed over ten-weeks leaving a depressed scar. Desferrioxamine was restarted again with care to replace the needle every 72 hours and PG has not recurred over the last 2 months.

PG is an autoimmune ulcer and is associated with a systemic disease in about 50% of patients. It is known to be associated with various haematological disorders including acute myeloid leukaemia, multiple myeloma, lymphoma or monoclonal gammopathy of undetermined significance. Trauma is a known precipitant of PG and it may have played a role in our patient. Therapeutic efficacy of topical tacrolimus 0.1% (Protopic) in the treatment of PG with associated myelodysplasia has not been widely reported. Conventional systemic immunosuppressive therapy may be hazardous in this group of patients who are often elderly.

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#### High dose cytarabine (HD-AraC) and cerebellar toxicity – a single centre experience

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In acute myeloid leukaemia (AML) standard dose AraC in combination with an anthracycline is part of induction. A 15–30-fold AraC dose escalation can elicit a therapeutic response in patients who failed treatment. Apart from potent myelosuppression, the dose limiting toxicity of HDAC is cerebellar damage. We report three AML patients who developed Cerebellar toxicity with use of HDAC at a single institution between June 2002 and June 2006.

*Patient 1:* A 37-year woman with AML had second relapse post an unrelated donor allograft and received HDAC. She developed mild cerebellar signs on day 5 and cytarabine was stopped. CT and MR of brain showed only a mild degree of cerebral atrophy and no other focal lesions. She died of TTP and GVHD with persistent ataxia.

*Patient 2:* A 62 year man with AML received HDAC for first relapse. He developed severe cerebellar signs on day 5 of treatment and required haemodialysis for renal failure. CT scan of brain was normal. He died of sepsis with persistent cerebellar toxicity.

*Patient 3:* A 51 year old man with AML received conventional chemotherapy and consolidation with MidAC and had intermittent diplopia 2 weeks later. He was admitted 2 months after with cerebellar signs. CT, MR head were both normal as was CSF. He continued to deteriorate, had status epilepticus and remains in vegetative state. No cause has been found.

*Conclusion:* We report two cases of severe neurotoxicity in patients treated with HDAC for relapsed AML and one patient experienced severe late neurotoxicity. The main risk factors for neurotoxicity of HDAC are dose, age of patient and renal impairment. Our experience suggests that stopping AraC once signs develop may not help to prevent progression. Therefore, caution needs to be exercised before

using HDAC especially in high-risk patients to prevent this complication. High dose AraC should not be used in patients with renal impairment.

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#### Frequent relapse of acute myeloid leukaemia by mitotic recombination

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Relapse is the commonest cause of death in acute myeloid leukaemia (AML), but the mechanisms leading to relapse are unclear. Recently, acquisition of uniparental disomy (UPD) by mitotic recombination (MR) have been reported in 15–20% of AMLs at diagnosis using whole genome single-nucleotide polymorphism (SNP) arrays. These abnormalities are cytogenetically invisible and are associated with homozygous mutations in several malignancies. Clonal evolution from heterozygous to homozygous mutations by MR could provide a mechanism for relapse. DNA from 27 pairs of diagnostic and relapsed AML samples were analysed using Affymetrix 10K SNP arrays. Copy number and loss of heterozygosity were analysed using in-house software. Regions of deletion, amplification and UPD were documented and compared between diagnosis and relapse. UPDs were acquired at relapse in eleven AMLs (30%). Six of these were UPDs of chromosome 13q, which lead to a change from heterozygosity to homozygosity for internal tandem duplication of FLT3 (FLT3 ITD). A further AML acquired UPD of 19q which lead to homozygosity for a CEBPA mutation 957 C to T. Three more AMLs had evidence of a subclone with UPD of 13q. One AML acquired UPD of chromosome 4q, for which a mutation has not been discovered. Acquisition of UPD by mitotic recombination is a frequent mechanism for relapse in AML. It is possible that targeting the associated homozygous mutations could treat or prevent relapses.

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#### The platelet-derived growth factor receptor beta fuses to two distinct loci at 3p21 in imatinib responsive chronic eosinophilic leukaemia

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We have identified three patients who presented with BCR-ABL negative myeloproliferative disorders and a *t*(1;3;5)(p36;p21;q33) or *t*(3;5)(p21-25;q31-35). Fluorescence *in situ* hybridization indicated that the platelet-derived growth factor receptor beta gene (PDGFRB) was disrupted in all three cases. 5' rapid amplification of cDNA ends (5'RACE) for the *t*(1;3;5) case identified an in-frame mRNA fusion between WDR48 at 3p21 to PDGFRB. Cases 2 and 3 were negative for WDR48-PDGFRB but instead harboured an mRNA fusion between GOLGA4 and PDGFRB. Imatinib, a known inhibitor of PDGFRbeta, selectively blocked the growth of *t*(3;5) myeloid colonies and produced clinically significant responses in all three patients. Strikingly, both GolginA4 and Wdr48 are involved with endocytic pathways and protein trafficking within the cell, a functional overlap with the previously identified Pdgfrb fusion partner rabaptin-5. Endocytosis is a well-defined mechanism for the attenuation of proliferative signalling by surface receptors and it is possible that the transforming ability of these fusions may depend, at least in part, directly on interference with cellular trafficking.

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### CCN3 reduces the clonogenic potential of BCR-ABL + cells

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Chronic Myeloid Leukemia (CML) is characterized by expression of the constitutively active BCR-ABL tyrosine kinase. Previously, we have identified down-regulation of the negative growth regulator, CCN3, as a result of BCR-ABL kinase activity and detected reduced CCN3 expression in human CML cell lines and primary human CML cells. We now report the growth inhibitory effect of CCN3 expression in human CML cells.

Colony formation assays were performed over 7 days to determine clonogenicity of CML cells expressing CCN3 and compared to cells treated with Imatinib (1 µM). Human K562 cells were transfected with vector alone or vector containing CCN3 using Amaxa nucleofactor technology or treated with Imatinib, for 24 hours prior to plating in methyl cellulose cultures. Increased CCN3 expression in K562 cells significantly reduced colony formation by  $65.4\% \pm \text{SD } 18.8$  when compared to cells transfected with vector alone ( $P = 0.027$ ,  $n = 3$ ). Treatment with Imatinib also reduced colony formation ( $75\% \pm \text{SD } 8.2$ ;  $P = 0.001$ ,  $n = 3$ ) compared to untreated cells. We next assessed the clonogenic effects of CCN3 and Imatinib on primary human CD34 + progenitor cells derived from CML peripheral blood samples at diagnosis ( $n = 3$ ). Cells were treated with exogenous addition of CCN3 (1 nM) or Imatinib (1 µM) for 24 hours prior to plating in methyl cellulose. CCN3 reduced clonogenic capacity by  $25.5\% \pm \text{SD } 3.9$  ( $P = 0.011$ ) whilst treatment with Imatinib reduced colony formation by  $37.9\% \pm \text{SD } 19.9$  ( $P = 0.010$ ).

CCN3 is known to be a negative growth regulator and increased expression of CCN3 in BCR-ABL + cells decreases cell clonogenic potential. Thus CCN3 down-regulation mediated by BCR-ABL offers growth advantage to hematopoietic cells.

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### Molecular responses to dasatinib reveal multiple mechanisms of imatinib resistance in patients with chronic myeloid leukaemia

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Resistance to Imatinib mesylate (IM) can develop in patients with chronic myeloid leukaemia (CML) through several mechanisms. Dasatinib, a dual specificity SRC/ABL kinase inhibitor that binds to the active conformation of ABL enabling *in vitro* inhibition of most IM-resistant mutants, can induce haematological and cytogenetic responses in IM-resistant CML patients. Molecular and cytogenetic responses to Dasatinib were evaluated in a cohort of IM-refractory or IM-intolerant CML patients in chronic phase ( $n = 11$ ) or accelerated phase/blast crisis ( $n = 5$ ), initially treated with either 100 or 140 mg

Dasatinib daily. PB BCR-ABL RQ-PCR and BM cytogenetics/FISH were performed at start of treatment and at three monthly intervals (follow-up 1–21 months). Mutation screening was performed by DHPLC followed by allele-specific PCR for the Dasatinib resistant T315I and sequencing to characterise other mutations. Fourteen patients had evaluable cytogenetic results. Six had a major cytogenetic response: four achieved complete cytogenetic response (CCR); two had partial responses. One patient had a minimal response. Of those attaining CCR, three had a major molecular response with one patient achieving a one log depletion of BCR-ABL transcripts. DHPLC analysis of 6/7 patients with cytogenetic responses indicated mutations of the ABL kinase domain in five. One further patient with a G250E achieved a one log reduction in BCR-ABL transcripts after two months of Dasatinib. Of the nine patients with no cytogenetic or molecular response to Dasatinib, the T315I was detected in two, BCR-ABL over-expression with sustained high BCR-ABL/ABL ratios  $>150\%$  detected in three and clonal evolution with gene amplification due to an extra Ph + observed in one. Dasatinib therefore elicited a cytogenetic and/or molecular response in seven patients by potentially overcoming ABL kinase domain mutation induced resistance or by inhibition of SRC-mediated signalling. In those CML patients without a molecular response, alternative processes causing IM resistance are implicated that require further elucidation.

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### Hypereosinophilic syndrome: challenges in diagnosis and management

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Hypereosinophilic syndrome is a rare heterogeneous condition. Recent developments in our understanding of the pathogenesis of this syndrome has led to diagnostic pathways, prompting potential subclassification into myeloproliferative and lymphoproliferative variants. We would like to present three cases of hypereosinophilic syndrome which demonstrate its complexity, and that management should be individually tailored. Case 1: A 55-year-old man presented with a necrotic skin rash and marked eosinophilia unresponsive to steroids. He developed splenomegaly and multiorgan failure, requiring months in the intensive care unit. He was unresponsive to imatinib and required treatment with cytarabine, hydroxycarbamide and alpha interferon to control his disease. He made a good recovery and is currently not on cytotoxics. Case 2: A 55-year-old woman with progressive severe respiratory compromise associated with eosinophilia and widespread low volume lymphadenopathy. This responded to steroids but she developed a desquamative rash and eosinophilia, which required further steroids. Her symptoms and blood counts are stable on alpha interferon. Case 3: A 36-year-old man who presented with severe eosinophilic cellulitis comprising more than 40% of his body surface area. This responded to steroids but new skin lesions developed on its cessation. He was unable to tolerate methotrexate or alpha interferon and is asymptomatic with normal counts on low dose hydroxycarbamide. All cases had bone marrow biopsies that were hypercellular with no maturation abnormalities within the myeloid, lymphoid or eosinophilic lineages. FIP1L1 PDGFR alpha mutations were not detected and cytogenetics were normal. We were unable to easily subclassify these patients. The first case illustrates the need for prompt treatment with cytotoxics in

some severe cases before a formal diagnosis is made. These cases highlight the difficulties in the diagnosis and management of such patients and that treatment modalities used will depend on the individual patient.

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**Analysis of the regulation of angiogenesis in the bone marrow of myelodysplastic syndrome transforming to overt leukaemia compared to acute myeloid leukaemia**  
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Dysregulation of angiogenesis, the formation of new capillaries from pre-existing vessels, increases bone marrow microvascular density (MVD) through a similar process as occurs in solid tumours, which display hypervascularity upon growth, facilitating the proliferation of the malignant clone.

In order to investigate the regulatory mechanisms controlling angiogenesis in the development of myelodysplastic syndromes

(MDS) and its progression to overt leukaemia (OL), bone marrow samples from control, paired samples from MDS patients before and after transformation to OL (MDS→OL) and *de novo* AML were analysed using immunohistochemical staining of vascular associated antigens, to visualise the microvasculature, to enable measurement of MVD, and quantitative polymerase chain reaction (PCR) to measure angiogenic mediator gene expression.

Immunohistochemical staining of the vascular associated antigens revealed significant increase of MVD in MDS and *de novo* AML compared to controls. Surprisingly, the MVD in MDS significantly decreased upon transformation to OL, which was also significantly lower than MVD of *de novo* acute myeloid leukaemia (AML). These findings were strengthened by the pattern of angiogenic mediator gene expression evaluated by quantitative polymerase chain reaction, which correlated with MVD, confirming the importance of various angiogenic mediators including VEGF, bFGF, TNF $\alpha$ , HGF and the angiopoietin mediators Ang-1 and Ang-2, as well as the angiogenic mediator receptors VEGFR2 and TIE2. Conversely, TGF $\beta$ , an angiogenic mediator, exhibited significantly higher expression in the bone marrow of MDS when OL developed, indicating the importance of this cytokine as the suppressive factor of angiogenesis in MDS→OL.

These findings indicate that although morphologically similar, the bone marrow microenvironment and pathogenic events occurring in MDS→OL and *de novo* AML differ remarkably, suggesting that anti-angiogenic therapy would display differing efficacy between *de novo* AML and leukaemia secondary to MDS.

## Poster Presentations: Nursing

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**Effect of selected healthy and psychosocial aspects on quality of life in adult patients with acute myeloid leukaemia undergoing autologous progenitor stem cell transplantation**

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**Background:** Study analyses the effect of selected healthy and psychosocial aspects of quality of life in adult patients with acute myeloid leukemia undergoing autologous progenitor stem cell transplantation at the Department of Clinical Haematology of the 2nd Internal Clinic of Charles University Hospital in Hradec Kralove, Czech Republic.

**Patients and methods:** The total number of respondents with acute myeloid leukaemia undergoing autologous transplantation from 2001 to 2003 was 19. The return rate of questionnaires was 63% (12 respondents). There were 100% ratable questionnaire. The mean age of all patients was 47.5 years old (age range 27–68). The males were seven and the females were five. The Czech version of an international generic European Quality of Life Questionnaire – Version EQ-5D was used. The effect of selected aspects (age, sex, level of education, marital status, number of associated diseases, smoking abuse, religion and time lapse from autologous transplantation on quality of life in patients was determined by means of analysis of variance.

**Results:** The above-mentioned factors proved statistically significant dependence quality of life (EQ-5D score and EQ-5D VAS) on age ( $P <$

0.01), religion ( $P < 0.05$ ), smoking abuse ( $P < 0.01$ ), education ( $P < 0.05$ ) and number of associated diseases ( $P < 0.05$ ). EQ-5D score (dimensions of quality of life) and EQ-5D VAS (a subjective health condition) significantly decrease with increasing age, religion, smoking abuse, level of education and number of associated diseases in patients with acute myeloid leukaemia undergoing autologous progenitor stem cell transplantation. The effect of other aspects on quality of life was not proven as statistically significant.

**Conclusion:** The global quality of life in adult patients with acute myeloid leukemia undergoing autologous progenitor stem cell transplantation is on greatly good level (mean EQ-5D score 75.1%, mean EQ-5D VAS 67.5%) at the Department of Clinical Hematology of the 2nd Internal Clinic of the Charles University Hospital in Hradec Kralove, Czech Republic.

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**Quality of life in patients with multiple myeloma and malignant lymphoma after autologous progenitor stem cell transplantation: an effect of selected psychosocial and healthy aspects on quality of life: a retrospective study**

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**Background:** The study analyses the effect of selected psychosocial and healthy aspects on quality of life in patients with multiple

myeloma and malignant lymphoma after the autologous progenitor stem cell transplantation. Patients and Methods: The total number of respondents after the transplantation from 2001 to 2003 was 80 and the return rate of questionnaires was 70% (56 respondents: 32 respondents – 18 male and 14 female with multiple myeloma and 24 respondents – 11 male and 13 female with malignant lymphoma). The average age of patients with multiple myeloma was 60 years and the average age of patients with malignant lymphoma was 44.5 years. The Czech version of an international generic European Quality of Life Questionnaire – Version EQ-5D was used. The effect of selected aspects (age, sex, level of education, marital status, number of associated diseases, smoking abuse, religion, type of disease and the time lapse from the transplantation) on quality of life in patients was determined by means of analysis of variance. Results: The above-mentioned aspects proved statistically significant dependence of quality of life on age, smoking abuse in patients with multiple myeloma and on type of disease. EQ-5D score (dimensions of quality of life) and EQ-5D VAS (a subjective health condition) significantly decrease with increasing age in both groups patients and with smoking abuse in patients with multiple myeloma, and are significantly higher in patients with malignant lymphoma. The effect of other aspects on quality of life was not proven as statistically significant. Conclusion: The quality of life in patients with multiple myeloma after the autologous progenitor stem cell transplantation is lower (mean EQ-5D score 68.9%, mean EQ-5D VAS 66.6%) than in patients with malignant lymphoma after the transplantation (mean EQ-5D score 82.7%, mean EQ-5D VAS 76.7%) at the Department of Clinical Hematology of the 2nd Internal Clinic of Charles University Hospital in Hradec Kralove, Czech Republic.

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### The role of admission flowcharts in ensuring rapid and appropriate access to inpatient facilities

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Advances in treatment for haematological malignancies have resulted in more patients with increasingly complex health needs requiring treatment as inpatients. As a level four centre, with referrals from all over South and Mid Wales, demand for beds can often exceed capacity; however, GPs and local hospitals can be understandably nervous to treat Acute Haematology patients.

When patients telephone the ward for advice, they may speak to a relatively inexperienced nurse who may feel unable to advise them not to attend for inpatient treatment, even if the problem is not directly related to their haematological problem.

The senior clinical nurses within the haematology directorate met to discuss how to manage this problem as well as how to ensure that nursing staff feel supported in the advice they give. The result of this meeting was a 'flowchart' to help to establish whether a patient requires immediate admission to the ward or can be reviewed initially by his GP or local hospital. Accompanying the flowchart was a list of questions to ask the patient before offering any advice, as well as who to contact for further advice about the appropriate management of the patient. This poster gives an overview of the problem and will demonstrate the flowchart and protocol for admission.

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### An investigation to assess the feasibility of nurse prescribing of blood components

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Currently prescribing blood components is viewed as a medical responsibility. The rationale for this is unclear, as blood components are not considered to be medicinal products. Lately, there has been increasing interest by nurses wishing to provide more seamless care for their patients in undertaking this role. The Department of Health guidance however, state that this is inappropriate for nurse prescribing. The National Blood Service (NBS) and the Scottish National Blood Transfusion Service (SNBTS) undertook a collaborative project to explore the feasibility of nurses having the right to prescribe blood components.

As a first step, a UK-wide survey to identify current practice and canvass the opinion of nurses and doctors was undertaken. The survey identified that 60% of respondents were supportive of nurses undertaking this role, citing it would have a positive impact on the quality of patient care, less treatment delays with doctors and nurses being able to use their time more effectively. The remaining 40% of respondents had reservations; these related to constraints with time, resources and worries around undermining medical care and responsibility.

In the light of the evidence from the survey and investigation, three proposals for the future direction of prescribing practice have been suggested:

*Proposal 1:* No change to the current situation.

*Proposal 2:* Determine if blood components could be classified as a borderline substance and included in the British National Formulary.

*Proposal 3:* Determine whether blood components could be classified as a therapy.

The ambiguous situation surrounding the classification of blood components is an issue, and will influence which proposal is taken forward. Due to positive feedback the investigators will continue to promote debate and lobby for support. Further development of non-medical prescribing to include the right for nurses to prescribe blood components has the potential to deliver a more patient-centred quality service.

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### Thalidomide audit

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Purpose of this audit was to assess the use of thalidomide, response rate to thalidomide, thromboembolic complications and to identify the regimen that produced the best response in myeloma at Russells Hall Hospital.

*Standard:* Thalidomide used as a second or subsequent line unless in trial.

Expected VTE <5% as single agent, 10–50% in combination [Jeffrey A Zonder – Haematology 2006].

Response rate as per ASH Education programme book 2005, page 358.

We retrospectively reviewed the case notes of patients between 2000–2005.

We enrolled 46 patients [42 Myeloma, 4 patients MPD/Myelofibrosis]. Twenty-eight per cent [13/46] received thalidomide as first line. This included nine myeloma patients of which 7/9 were in trial (77.7%). 72% (33/46) received thalidomide as Second line.

Twenty-two patients experienced side effects and four stopped thalidomide. Main side effects were neuropathy and constipation predominantly grades 1 and 2. DVT was seen in two patients.

We used EBMT criteria to assess the response. In relapsed/refractory Myeloma 66.6% (22/33) responded to thalidomide, 68% in combination as CTD and 32% as single agent. Twenty-one per cent (7/33) achieved CR, 39% (13/33) achieved PR and good PR. When used as first line 88% responded (77% CR and good PR [6/9], 11% PR [1/9]). Response rate was 50% (4/8) in refractory myeloma.

**Best response:** When thalidomide used as first line response rate was 77.7% (7/9) vs 55.5% (5/9) with VAD regimen. As a second line in relapsed/refractory myeloma thalidomide regimen had a response of 42.4% (14/33) vs 33.3% (2/6) with VAD regimen.

**Conclusions:** Majority of first line treatment are on trial (77.7%). Peripheral neuropathy and constipation are common side effects (grade 1–2). Thromboembolic complication is 4%, less than the expected rate and in combination regimen.

66.6% responded to thalidomide in relapsed/refractory myeloma. 50% of patients with refractory myeloma have responded to thalidomide. 88% responded to thalidomide when used as first line.

Thalidomide containing regimen seemed superior to VAD/C-VAD regimes (77.7% vs 55.5% as first line and 42.4% vs 33.3% as second line).

## Poster Presentations: Paediatrics

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### Reduced intensity conditioning transplant as a successful treatment for Kostmann's syndrome J Thachil\*, T Dutt\*, P Bolton-Maggs†, M Caswell\* and R Keenan\*

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Kostmann syndrome (KS) is a congenital disorder characterized by severe neutropaenia ( $< 0.2 \times 10^9/l$ ) and recurrent bacterial infections. G-CSF improves neutropaenia in 90% of cases. For those who do not respond, stem cell transplantation is the only effective treatment.

We describe a boy with KS presenting with recurrent life threatening infections including pneumonia and scrotal abscesses. No response was observed to escalating doses of G-CSF up to  $160 \mu g/kg/day$ .

We considered his transplant options. Eight patients with KS on the Severe Chronic Neutropaenia International Registry underwent transplant between 1976 and 1998, one received a non-myeloablative regimen with cyclophosphamide alone but rejected the graft. Only three patients received unrelated donor transplants, two died and the third, though engrafted, was noted to have severe failure to thrive. The French Registry reports nine patients between 1993 and 2003, all but one receiving preparatory myeloablation. The non-myeloablative regimen consisted of fludarabine and anti-thymocyte globulin before a second stem cell transplant. The patient failed to engraft and died.

Our patient did not have a sibling and it was decided to proceed with matched unrelated donor, peripheral blood stem cell transplant. We opted to employ a novel reduced intensity conditioning regime. (fludarabine  $30 mg/m^2$  from day -8 to day -5, Campath-1H  $0.2 mg/kg$  from day -6 to day -2 and thiopeta  $250 mg/m^2$  from day -4 to -2.)

The patient successfully engrafted with a neutrophil count greater than  $0.5 \times 10^9/l$  on day 12. Peri-transplant toxicity was low with only one febrile episode. The patient is now twenty-two months post transplant with full donor chimaerism, maintains a normal neutrophil count and has not required hospitalisation post transplant.

We propose that a reduced intensity conditioning transplant from a matched unrelated donor is an effective treatment for Kostmann syndrome, and should be considered for those patients who fail to respond to G-CSF.

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### The impact of infection in early childhood on intellectual function in adolescence: evidence from children with sickle cell disease

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**Background:** Evidence obtained from older adults with neurodegenerative disorders suggests that systemic infection promotes an inflammatory (cytokine) cascade that can lead to an exacerbation of brain pathology and cognitive decline. Infection as a risk factor for neurocognitive deficit in sickle cell disease (SCD) has previously received little attention, although there is some evidence to hypothesise that a similar pathophysiological pathway may be activated in these children.

**Methods:** We retrospectively examined multiple steady-state white blood cell (WBC) values from 52 children with HbSS SCD (0–16 years) to explore the influence of age, and the association with level of intellectual function (Wechsler Full Scale IQ). Data were subjected to mixed-model analysis of repeated measures.

**Results:** WBC count was chronically high during early to mid childhood, but decreased thereafter and was maintained at a lower level through adolescence. In support of our hypothesis, every IQ point lost in late childhood (9–16 years) was associated with a .14 increase in WBC count during early childhood (0–9 years) (95% CI, 0.26–.02,  $P = 0.022$ ). While the extent of WBC count variability was not fully related to later IQ ( $P = 0.137$ ), approximately 30% of its variance between children during the ages of 0–9 years was associated with the level of intellectual function that they demonstrated in later childhood.

**Conclusion:** These pilot data suggest that systemic infection may contribute to intellectual losses in children with SCD. Further research is required to confirm the possibility that systemic infection exacerbates underlying brain vulnerability via cytokine activation.

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### Changes in brain magnetic resonance imaging findings in children with sickle cell disease

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Children with sickle cell disease (SCD) are at high risk for both neurologically overt cerebral infarcts associated with stroke and neurologically silent cerebral infarcts (SCI) correlated with neurop-

sychometric deficit. We performed magnetic resonance imaging (MRI) & angioigraphy (MRA) on unselected group of 42 children with SCD aged 2.5–19 years to assess the prevalence of SCI. 19 children had abnormal imaging (45%). Four children had had overt stroke (3 females, 1 male), 2 had SCI with stroke (50%). Fifteen (38%) children had evidence of SCI (9 females, 6 males) aged <5 years: 2, 5–10: 7, 10–15 3, >15 years: 3. This baseline prevalence is significantly more than previously reported. Few centres that have looked at this as a screening tool. Ninety-three per cent of children with SCI had lesions in the frontal cortex. Those with overt stroke had lesions of frontal and parietal cortex. All the patients with overt stroke are on a transfusion programme. Repeat MRI/MRA did not reveal any new SCI, suggesting benefit of chronic transfusion. None of our patients with SCI have developed overt stroke. The investigation has

identified two children with SCI on MRI/A and elevated TCD, who have been commenced on a prophylactic transfusion programme.

Elevated TCD is known to predict of increased stroke risk and there is evidence to suggest that SCI are associated with an increased stroke risk. Studies have shown discordance in MRI and TCD findings, suggesting that they reveal different aspects of CNS injury in patients with SCD. More specific indicators of early CNS pathology, such as neuropsychometric testing may be beneficial. Chronic transfusion decreases SCI and stroke risk in children with elevated TCD, further study is required to determine its risk-benefit ratio in children with SCI detected on MRI/A. We are incorporating this investigation into our surveillance of children with SCD and will be prospectively evaluating MRI/A alongside TCD and neuropsychometric testing.

## Poster Presentations: Red Cell Disorders

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### The use of continuous positive airways pressure in the management of acute chest syndrome in sickle cell disease

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Acute chest syndrome (ACS) is the second most common cause of hospital admission in patients with Sickle Cell Disease (SCD) and is the leading cause of mortality in young adults. In our centre we use early ventilatory support with Continuous Positive Airways Pressure (CPAP) in patients who are markedly hypoxic or have ACS, in addition to standard therapy with intravenous fluids and antibiotics. We report our recent experience here.

Twenty-three episodes of ACS were identified retrospectively over a two year period and the notes of those patients were reviewed. Eighteen patients were involved, as three patients had more than one episode of ACS during the study period. There were 10 female and eight male patients with an age range of 19–53 years (mean 28 years). The distribution of phenotype was representative of our clinic population consisting of 13 patients (72%) with homozygous sickle cell disease, three patients with HbSC disease and two patients with HbSBthalassaemia.

Chest pain was a feature in 19 of the episodes (83%), pyrexia was seen in 21 episodes (91%) and dyspnoea was a feature in 22 episodes (96%). Five patients did not have changes on their chest radiograph, but had respiratory symptoms and were markedly hypoxic. In only four episodes (17%) was a red cell transfusion required because of persistent hypoxia despite CPAP. No patient required mechanical ventilation and there were no deaths. These results compare very favorably with similar studies from the UK and the USA, with reduced requirements for both transfusion and mechanical ventilation seen in our study.

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### Plasma haemoglobin levels in Omani patients with sickle cell disease

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Intravascular haemolysis is common in sickle cell disease (SCD). The released Hb in plasma reacts readily with nitric oxide and limits its

bio-availability, and may promote vaso-occlusion as nitric oxide has potent vaso-dilatory properties. Symptomatic SCD is common in Oman where the gene frequency is 6%. The plasma Hb levels of 16 adult Omani patients with sickle cell disease, with frequent crises (more than three vaso-occlusive crises per year requiring hospitalization) were compared to 17 patients who were stabilized with infrequent crisis with or without hydroxyurea treatment. Plasma Hb levels in patients with frequent crises was  $257.9 \pm 87.2$  mg/l (105.0–370 mg/l) compared to  $190.8 \pm 60.7$  mg/l (74.0–310.0 mg/l) in stabilized patients without frequent crisis, and this represented a significantly difference in the two groups ( $P = 0.041$ ). Although overall WBC count was high in the frequent crisis group ( $11.79 \pm 4.82 \times 10^9/l$  compared to  $8.60 \pm 4.32 \times 10^9/l$ ), however, this did not represent a significantly difference between the two groups ( $P = 0.066$ ). Lower Hb levels, which was statistically insignificant, were also seen in the frequent crisis group ( $8.69 \pm 1.55$  g/dl compared to  $10.13 \pm 2.06$  g/dl in the infrequent crisis group;  $P = 0.086$ ). The neutrophil count was significantly higher ( $P = 0.012$ ) in the frequent crisis group compared to those on hydroxyurea and with infrequent crisis ( $7.04 \pm 3.67 \times 10^9/l$  and  $4.31 \pm 3.02 \times 10^9/l$ , respectively). Free-plasma Hb was positively correlated to the WBC count ( $r_s = 0.451$ ,  $P = 0.011$ ) and overall monocyte count ( $r_s = 0.553$ ,  $P = 0.001$ ). These findings may be useful in defining the role of haemolysis in the morbidity of sickle cell disease.

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### High definition contrast-enhanced MR imaging in paroxysmal nocturnal haemoglobinuria (PNH) suggests a high frequency of subclinical thrombosis

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Thrombosis is the most feared complication in PNH and is reported to occur in >40% of patients. Proposed mechanisms of thrombosis include depletion of nitric oxide (NO) by intravascular haemolysis and increased activation of PNH platelets. The occurrence of

subclinical thrombosis in PNH patients has not been previously studied using modern imaging techniques. In order to assess for subclinical thrombosis we evaluated PNH patients with a comprehensive state-of-the-art MRI protocol (using both blood pool and conventional Gadolinium based contrast agents) for the detection of subclinical thrombosis and its sequelae. The protocol consisted of: (a) lung perfusion and pulmonary MRA, (b) cardiac MR-including quantitative studies of both ventricles, right heart flow dynamics and delayed enhancement for detection of left ventricular damage, (c) abdominal MR for the assessment of hepatic and portal venous systems and kidneys. Ten PNH patients (median age 31.5 years) with large PNH clones but without previous clinical evidence of venous or arterial thrombosis underwent imaging. Five (50%) patients were on primary anticoagulant prophylaxis. There was evidence of significant renal haemosiderosis throughout the cortices in 9/10. Two patients had small myocardial scars suggestive of previous unsuspected ischaemic damage. Six patients had sub-segmental perfusion defects mainly in the peripheries of the lung fields indicative of previous small pulmonary emboli. No such subclinical thromboses would be anticipated in an age-matched control population. Eight patients had reduced right ventricular ejection fractions (mean  $42.2\% \pm 1.8\%$ ; normal range 48–63%). The plasma B-type natriuretic peptide (BNP) level was high in all 10 patients (median  $29.4 \text{ pmol/l}$ ; range 18.7–373.90; normal subjects  $4.89 \pm 1.00 \text{ pmol/l}$ ). BNP has been shown to increase in proportion to right ventricular dysfunction in pulmonary hypertension. No intra-abdominal defects were identified with the current protocol. In summary, we identified abnormalities suggestive of previous subclinical thromboses in six of 10 haemolytic PNH patients by high-resolution MR imaging, including in patients on primary prophylaxis with warfarin. Effective prevention of thrombosis is an important aspect of the therapy in PNH.

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### The incidence and prevalence of paroxysmal nocturnal haemoglobinuria (PNH) and survival of patients in Yorkshire

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PNH is characterised by a population of blood cells deficient in glycosylphosphatidylinositol-linked proteins resulting in the features of intravascular haemolysis and thrombosis. PNH is a rare disorder, but its incidence and prevalence have been poorly defined. In order to better define the incidence and prevalence, data was collected on PNH patients in the strategic health authorities of North and East Yorkshire, Northern Lincolnshire and West Yorkshire between January 1991 to July 2006. All patients were diagnosed by flow cytometry at the HMDS laboratory. The population of the study region is 3 742 835. Seventy-six PNH patients were diagnosed giving an incidence of 0.13/100 000/year. Based on incidence and survival rates, the estimated 15-year prevalence of PNH is 1.59 per 100 000 resulting in a predicted prevalence of 59 patients in the study region. A neutrophil clone size  $> 50\%$  is a predictor of increased thrombotic risk; this study predicts 25% of patients will have  $> 50\%$  PNH neutrophil clone size, 43% with  $> 10\%$ , and 82% with  $> 1\%$ . Platelet count  $> 100 \times 10^9/\text{l}$  is used to consider primary prophylactic anticoagulation if the neutrophil clone size is  $> 50\%$ . In this study, the platelet count is  $> 100 \times 10^9/\text{l}$  in 32% and  $< 30 \times 10^9/\text{l}$  in 27%. Levels of LDH were elevated in 82.5% of patients. Of the 59 patients in the study region, 33% reported haemoglobinuria. Overall survival was 78% with a median follow-up of 6.25 years (range 0–15 years). Survival was compared between patients with (a) haemoglobinuria

(89%) vs those without (76%); (b) neutrophil clone size  $> 50\%$  (81%) vs  $< 50\%$  (80%) and (c) platelet counts  $< 30 \times 10^9/\text{l}$  (61%) vs  $> 30 \times 10^9/\text{l}$  (86%). Worse survival was predicted by platelet counts  $< 30 \times 10^9/\text{l}$  ( $P = 0.008$ ). With a population of 5 710 537, Britain should have an estimated 75 new cases of PNH/year and a predicted prevalence of 908 patients. This study is the first to accurately report the incidence and prevalence of PNH in a given population in a well-defined geographical area.

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### The use of a text messaging service to improve attendance at a sickle cell clinic

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The attendance rates at out-patient clinic appointments tend to be poor within the sickle cell population. This is partly because patients forget their appointments which are usually at 6–12-month intervals. We attempted to decrease the non-attendance rates in our paediatric and adult clinics using a mobile phone texting service to remind patients of their routine clinic appointments. This was piloted from July to December 2006 and a reminder message was texted to patients or parents of the patients due to attend the clinic that week.

From January to June 2006, 67% of paediatric and 55% of adult patients attended their clinic appointments. This compares to attendance rates of 72% in the paediatric clinic and 62% in the adult clinic from July to December 2006, after the texting service was introduced. This showed an overall improvement in attendance, although there was better attendance at the paediatric clinic. Statistical analyses showed significant improvements in attendance and non-attendance rates across both clinics. There was a highly significant improvement in the adult non-attendance rate ( $P < 0.005$ ), and significant improvements in paediatric attendance ( $P < 0.01$ ) the paediatric non-attendance rate ( $P < 0.05$ ) and adult attendance ( $P < 0.05$ ).

We also noted that we did not have up-to-date mobile phone numbers for all patients; overall 58% of all patients who had clinic appointments were texted, the remaining 42% were not texted reminders because we did not have their current mobile numbers. We are now collecting current mobile numbers from all patients when they attend clinic and expect to see further improvements in attendance in the future.

This study shows that the texting service is feasible and is contributing to improvements in clinic attendance at Central Middlesex Hospital. Additionally the service has been received positively by patients, who have reported finding it very helpful in reminding them of their appointments.

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### The use of real-time PCR technique in the classification of thalassaemias

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The thalassaemias are a diverse group of disorders characterized by decrease in the amount of normal hemoglobin. Reduced synthesis of



one or more globin chains leads to imbalanced globin chain production which influences the severity of thalassaemia. The two main types of thalassaemias are alpha-thalassaemia caused by mutations in the gene coding for alpha globin and beta-thalassaemia resulting from beta globin gene mutations.

The aim of our study was to look for genetic changes in the alpha and beta globin genes in a group of unrelated Polish patients. We also discuss the effect of these mutations on the expression level of the globin genes.

Molecular analysis revealed eight different mutations affecting transcription and RNA processing (5'UTR + 33C>T; IVS-I-15T>C; IVS-II-745C>G; IVS-I-1G>A; IVS-I-5G>A; IVS-I-6>A; 1 and 2 substitutions in 3'UTR) and two structural mutations (Val98Met HbKoln; Phe42 del) in the alpha-globin gene. Multiplex PCR showed 3,7 deletion in alpha-globin gene cluster in patients.

To analyze gene expression we used real-time PCR. Pfaffl model was employed to estimate the relative changes in alpha-, beta-, gamma- and delta-globin genes expression of thalassaemia subjects in comparison with healthy individuals. Data normalization was carried out against two previously selected reference gene transcripts.

There is a correlation between alpha-globin and beta-globin mRNA levels and genetic background of the analyzed patients. Most of thalassaemia patients revealed increased gamma-globin and delta-globin mRNA levels but there is no correlation between gamma-globin and delta-globin gene expression levels and HbA2 and HbF levels.

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### Hereditary erythrocyte disorders in Poland

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In 2003, in response to a need to improve diagnosis of hereditary disorders of erythrocytes in Poland, we established at the Institute of Haematology and Transfusion Medicine a specialized diagnostic centre. Before that date most of those diseases were unrecognized, and some like thalassaemia were considered very infrequent. The only exceptions were erythrocyte enzymopathies but the diagnostic laboratory for those disorders ceased of action in 2002. We started with setting up modern diagnostic techniques and elaborating some of our own. The latter applies to developing of the method for determination of carbohydrate molar composition of membrane glycoproteins separated by SDS-PAGE, refining SDS-PAGE to determine major protein bands in terms of copy number per erythrocyte, and recently, procuring a rapid screening test for congenital dyserythropoietic anaemia type II. Since 2004 we analyzed 1800 blood samples from patients with anaemia of uncertain etiology, most of which were sent to us by mail from all over Poland. We have established correct diagnosis in 182 patients with spherocytosis, 64 patients with deficiency of erythrocyte enzymes (61 with G6PD, two with PK and one with GPI), 281 patients with beta thalassaemia minor, at least 27 patients with beta thalassaemia intermedia (two homozygotes for IVS1-6(T>C) mutation were identified) and four patients with congenital dyserythropoietic anaemia type II. In addition we found 11 patients with alpha thalassaemia (see Maciag et al, this Meeting). Most surprising was the high number of patients with beta thalassaemia. Of those patients 70% had mutations of the Mediterranean type. The most common

mutations were IVS1-6(T>C) and IVS2-745(C>G). Unfortunately, in a large percentage of cases we are yet unable to establish a diagnosis. More advanced techniques for a fast diagnosis of hereditary disorders of erythrocytes are needed.

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### Abnormalities of erythrocyte glycoconjugates are identical in two CDA-II families with different chromosomal localization of the disease gene

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Congenital dyserythropoietic anaemia type-II (CDA-II) is a rare, recessively inherited disease characterized among others by abnormalities affecting erythrocyte glycoconjugates. Here we report the carbohydrate molar composition of erythrocyte anion exchanger (AE1), glycophorin A, polyglycosylceramides as well as total carbohydrate contents of erythrocyte oligoglycosylceramides in two affected siblings and two healthy parents from each of the two CDA-II families: family 1 with the typical localization of the disease gene (CDAN2) to chromosome 20q11.2 and family 2 in which this localization was excluded and a detailed glycoconjugate analysis was never performed. In spite of the different genetics, the glycoconjugate abnormalities of erythrocytes in the two families were identical suggesting a complex inheritance of CDA-II. The glycoconjugate abnormalities in the parents showed a dosage effect thus, making possible detection of CDA-II carrier state regardless of chromosomal localization of CDAN2. We also report for the first time that erythrocyte AE1 protein is decreased in CDA-II homozygotes and obligate carriers alike.

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### Pulmonary arterial hypertension in children with sickle cell disease: how common is it?

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The prevalence of Pulmonary Arterial Hypertension (PAH) in adults with Sickle Cell Disease (SCD) is estimated to be 32% with a 2-year mortality of 50% from diagnosis. The age of onset is currently unknown. Echo Tricuspid Regurgitant jet Velocity (TRV) has been shown to correlate well with catheter measurements of pulmonary artery pressures and is useful as a non-invasive tool. Hyperhaemolytic state defined by Haemoglobin (Hb) < 8.5 g/dl and number of Acute Chest Syndrome (ACS) events may be associated with an increased risk. Peak incidence of ACS is between 2-5 years. **Aims:** To prospectively determine prevalence of PAH in children with SCD and define associated risk factors.

**Methods:** Fifty ( $F = 26$ ) patients with SCD, median age 14, range 10-18 year, and 50 ( $F = 25$ ) sex-, age-, race-matched healthy controls were recruited. TRV was measured using 2D-Doppler echo; all subjects were at baseline state of health. Patients were free of any vaso-occlusive crisis for >14 days. Hb was measured in all subjects; detailed medical history was taken with verification from hospital notes.

**Results:** Sixteen out of fifty (32%) of patients had TRV > 2.5 of m/s. 4/50 (8%) of controls had TRV > 2.5 m/s. Age vs TRV > 2.5 m/s  $P = 0.846$ ; Gender vs TRV > 2.5 m/s  $P = 0.071$ ; Hb < 8.5 g/dl vs TRV > 2.5 m/s  $P = 0.108$ ; ACS admission > 1 vs TRV > 2.5 m/s  $P = 0.163$ . The youngest patient with TRV > 2.5 m/s was 10 years. Intra and inter-observer repeatability/reproducibility studies were robust.

**Conclusion:** These data suggest a significant number of children with SCD have echo evidence of PAH. We were unable to identify association between elevated TRV and hyper-haemolysis, number of ACS events or increasing age within the patient group. All subjects with raised TRV will be followed up including the controls. We suggest all children with SCD be screened for PAH from age 5 years onwards. Longitudinal studies with therapeutic intervention are required to enable monitoring of disease progression and response to treatment.

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### A 16.5 kb deletion in the alpha globin cluster associated with an extremely mild phenotype

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We describe and characterise a *de novo* deletion in the alpha-globin cluster identified in a Vietnamese neonate and associated with an extremely mild alpha-thalassaemia phenotype. The 16.5 kb deletion removes a significant proportion of the alpha-globin cluster including pseudo zeta, alpha<sup>D</sup>, pseudoalpha1 and the alpha2 gene but importantly leaves the alpha1 gene and associated promoter completely intact. Located at the breakpoint junction is an insertion sequence which most likely originates from a repeat element in the pseudozeta gene. Results from functional analysis of the mutant chromosome in isolation suggest that this deletion may have an upregulatory effect on the intact alpha1 gene. This apparent upregulation could be related to the increased proximity of the alpha1 gene to *cis*-acting regulatory elements including HS-40 and/or reduced competition for them due to the absence of the dominant alpha2 gene and the minor alpha<sup>D</sup> gene. The observation that this novel deletion is associated with such a mild phenotype suggests that much of the DNA removed by this deletion is functionally redundant. Furthermore, it is entirely possible that similar types of deletions could exist that would remain undetected.

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### Single centre experience of the efficacy of screening for proliferative sickle cell retinopathy

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Visual loss is common in Sickle Cell Disease, affecting up to 20% of patients, and is usually due to proliferative sickle retinopathy (PSR). As the majority of patients with PSR are asymptomatic we provide yearly ophthalmology screening for all patients with SCD and offer pan-retinal photocoagulation laser treatment to those with PSR. We decided to assess the efficacy of this programme by reviewing all

ophthalmic clinic attendances over the previous two years, because of poor attendance at ophthalmology appointments and also because over 60% of patients identified with PSR show regression of the retinopathy over time without intervention.

By using the hospital information system we identified 256 patients who had been referred to the ophthalmologists of whom 140 attended their appointment. The notes were available for 71 patients (51%) and these were reviewed. Thirty-nine patients (55%) had no sickle retinopathy or non-proliferative retinopathy. No patients had grade 1, 13 had grade 2, three had grade 3, six had grade 4 and ten had grade 5 retinopathy. Eight out of the ten patients (80%) with grade 5 and three out of the five patients (60%) with grade 4 retinopathy had HbSC disease.

Eleven patients were symptomatic, nine with Hb SC and two with Hb SS and all of these had grade 3 retinopathy or above. Laser treatment was necessary in nine patients, all were symptomatic and eight had HbSC.

We have a poor rate of attendance to ophthalmology appointments in our patient group, leading to a waste of resources. We found a low incidence of proliferative retinopathy in patients with HbSS or HbSBthalassaemia. In order to maximize uptake of ophthalmology screening we should offer this as part of a one stop, annual clinic review and target the HbSC population for screening.

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### TRIUMPH, a randomized placebo-controlled phase III trial, demonstrates that the terminal complement inhibitor eculizumab improves anaemia in PNH

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Paroxysmal nocturnal haemoglobinuria (PNH) is a life-threatening haemolytic anaemia in which red blood cells (RBCs) lacking complement inhibitory proteins are sensitive to complement-mediated destruction. Intravascular haemolysis often requires transfusion with packed RBCs (PRBCs) to maintain tolerable haemoglobin levels. Eculizumab, a terminal complement inhibitor, has been shown in a placebo-controlled randomized phase III trial (TRIUMPH) to reduce haemolysis and transfusion requirements. We report here a detailed analysis of the effect of eculizumab on various parameters of anaemia in these study patients. Eculizumab-treated patients, as compared to placebo, showed an 85.8% decrease in intravascular haemolysis (as measured by LDH area under the curve,  $P < 0.001$ ), resulting in a 68% increase in PNH RBC mass from a mean of  $1.192 \pm 0.1 \times 10^{12}$  cells/l at baseline to  $2.007 \pm 0.13 \times 10^{12}$  cells/l at 26 weeks ( $P < 0.001$ ). The PNH RBC mass in placebo-treated patients was unchanged. Similarly, haemoglobin levels in eculizumab-treated patients increased relative to placebo ( $P < 0.001$ ). PRBC units transfused decreased from a median of 10.0/patient with placebo to 0.0/patient with eculizumab ( $P < 0.001$ ), and 51.2% of eculizumab-treated patients became transfusion independent (vs 0.0% of placebo patients,  $P < 0.001$ ). Even patients who required some transfusions while on eculizumab showed a marked reduction in transfusion requirement (from a median of

10.0 U/patient with placebo to 6.0 U/patient with eculizumab;  $P < 0.001$ ) as well as in intravascular haemolysis ( $P < 0.001$ ). The reduction in PRBC units transfused with eculizumab was observed regardless of transfusion requirements prior to treatment (4–14 U/year; 15–25 U/year; and  $> 25$  U/year,  $P < 0.001$  for each stratum). These data demonstrate that control of intravascular haemolysis in PNH with eculizumab results in a substantial improvement in anaemia, as evidenced by an increase in endogenous RBC mass and haemoglobin, and a reduction in transfusion requirements. These improvements with eculizumab are demonstrated regardless of historical transfusion requirements or whether patients achieve transfusion independence during treatment.

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### The terminal complement inhibitor eculizumab reduces thrombosis in patients with paroxysmal nocturnal haemoglobinuria

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Life-threatening thromboembolism (TE) is the most feared complication in paroxysmal nocturnal haemoglobinuria (PNH). Approxi-

mately 45% of PNH deaths result from TE. Primary prophylactic anti-coagulation may reduce the thrombotic risk in PNH patients, although controlled studies have not been performed and there is a risk of serious haemorrhage. Three completed studies of the terminal complement inhibitor eculizumab in PNH recently demonstrated dramatic reductions in intravascular haemolysis and units of red cells transfused. We prospectively examined the aggregate TE event rate in eculizumab-treated patients from these studies and a subsequent common phase 3 extension study. Before receiving eculizumab, 124 TE events in 195 patients were identified; 103 patients were on anti-thrombotics. Eculizumab reduced the TE rate in each study. The aggregate TE event rate with eculizumab treatment was 1.07 per 100 patient years, compared to 7.37 ( $P < 0.001$ ) in the same patients before eculizumab treatment, corresponding to a reduction of 85%. Sensitivity analyses showed the effect to be robust. With restriction of the pre-treatment observation period to the 12-months before initiation of the trials, the TE event rate with eculizumab was reduced 94% from 17.21 to 1.07, respectively ( $P = 0.002$ ). The TE event rate was reduced from 21.42 pre-treatment in patients with TE prior to the trials ( $n = 63$ ) to 2.27 during eculizumab treatment ( $P < 0.001$ ). Most TE events prior to eculizumab treatment occurred in patients receiving anti-coagulants, indicating that this therapy may be insufficient to prevent thrombosis. Of 103 patients on anticoagulants, there were 54 TE events in 30 patients over 385.73 patient years (14.00/100 patient years) pre-eculizumab compared to one TE event with eculizumab in 161.8 patient years (0.62/100 patient years;  $P < 0.001$ ), demonstrating that eculizumab reduces the risk of thrombosis in anticoagulated PNH patients. These data show that long-term eculizumab treatment results in a clinically and statistically significant reduction in thrombosis in patients with PNH.

## Poster Presentations: Transfusion

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### Platelet count has no influence on traumatic and bloody lumbar puncture in children undergoing intrathecal chemotherapy

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The British Society of Haematology (BSH) produced guidelines in 2002 recommending a platelet count of greater or equal to  $50 \times 10^9/l$  was necessary to safely proceed with a lumbar puncture (LP). Howard *et al.* (2000) reported a large retrospective analysis of 4309 LPs performed in 959 children including 941 procedures with platelet count of  $< 50 \times 10^9/l$  with no neurological haemorrhagic complications. This paper recommended to perform routine LP, a platelet count of  $10 \times 10^9/l$  or higher would be adequate.

We have retrospectively analysed our institute experience of 54 patients undergoing 713 LP procedures using greater or equal to  $30 \times 10^9/l$  as a cut off for a safe lumbar puncture. We have evaluated complications and determined whether the platelet count has any significance on the rate of red cell contamination. Of the 713 LPs, 65 (9%) were traumatic and 30 (4%) were bloody, but this was not

dependant on the pre LP platelet count. These results indicate a significantly lower rate of traumatic and bloody LPs than previously reported. There were no post LP bleeding or neurological complications.

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### Developing a blood conservation care plan for Jehovah's Witness patients with malignant disease

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Jehovah's Witnesses (JWs), like any other patients, seek the most effective treatments for malignant disease. Successful modern treatment often involves intensive chemo-radiotherapy or major surgery supported by blood component therapy. A care plan for JWs must take into account their informed refusal of the transfusion of whole blood and its primary components (red cells, platelets, granulocytes and plasma), accepting the additional risks this refusal

implies. This may prove challenging to clinical staff. However, lessons learned from JWs undergoing 'bloodless surgery' have been important in developing modern blood conservation strategies and there have been recent reports of patients surviving aggressive therapies such as remission induction for acute leukaemia and haemopoietic stem cell transplantation.

To help clinical teams develop appropriate care plans a draft guideline, or *aide memoire*, has been produced by JW Hospital Information Services and a number of experienced haematology and oncology clinicians. This document is presented for discussion and modification by delegates.

Key elements include understanding that the following may be acceptable to individuals: blood derivatives (e.g. albumin, cryoprecipitate), cell salvage, apheresis and haemopoietic stem cell procedures.

The guideline emphasises consideration of blood sparing strategies from the initial MDT meeting and constructive involvement of the local JW Hospital Liaison Committee. Possible surgical strategies include pre-operative optimisation of Hb, minimising surgical blood loss, cell salvage technology (with filtration or irradiation) and minimally invasive tumour embolisation or radio-frequency ablation. In medical therapies, the management of profound anaemia and thrombocytopenia are key and the appropriate use of growth factors, haemostatic agents and novel treatments with reduced myelosuppression warrant consideration.

Blood conserving care plans in this difficult area are feasible and in the light of potential blood shortages certain elements of such plans could be considered for general application.

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# **Cryosupernatant and solvent detergent fresh frozen plasma (octaplas) usage at a single centre in acute thrombotic thrombocytopenic purpura**

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Thrombotic thrombocytopenic purpura (TTP) is an acute, life threatening disorder and plasma exchange (PEX) remains the mainstay of treatment. Plasma therapy remains the mainstay of treatment in acute TTP episodes and until December 2005, apheresis was initiated with cryosupernatant unless patients had a previous severe allergic reaction or refractory disease, so continued with Solvent-Detergent Fresh Frozen Plasma (S/D FFP), Octaplas. We reviewed 50 acute TTP episodes involving 33 patients, primarily acute idiopathic ( $n = 25$ ). Thirteen episodes used cryosupernatant only and 15 episodes started with cryosupernatant and changed to Octaplas. Reasons for changing were young age ( $n = 1$ ), refractory disease, ( $n = 2$ ) and allergic reactions to cryosupernatant ( $n = 22$ ). Once Octaplas had been used, it was continued on further admissions. In 22 episodes, Octaplas was used exclusively. Cryosupernatant was used in 27.6% (total volume 508.25l) and Octaplas in 72.4% (total volume 1327.6l) of all episodes. The total number of plasma exchange procedures using cryosupernatant were 172 and Octaplas were 512. The number of citrate reactions and allergic (plasma) reactions were halved in those receiving Octaplas compared to cryosupernatant (10% vs 18% and 4.7% vs 9.8% respectively). There were 21 line infections and in approximately 70% of cases the infection was associated with a reduction in platelet count  $<150 \times 109/l$  and was primarily due to *Staphylococcus aureus*. In all 50 episodes, the only documented thrombosis was a superficial non-central vein. In episodes receiving only cryosupernatant or Octaplas, there was no significant difference in the median number of PEX to remission, 7

(3–14) and 8.5 (5–30), respectively. Baseline viral screen in all episodes was negative after discharge following an acute episode. In conclusion, cryosupernatant and S/D FFP (Octaplas) appear equally efficacious. However, the risk of allergic/urticarial and citrate reactions was more common with cryosupernatant. There was no documented viral transmission with either product or episodes of TRALI.

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# **An audit of red cell concentrate, fresh frozen plasma and cryoprecipitate use within the west of Scotland: a 5-year repeat analysis**

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The Regional Haematology Audit Group covers a population of 3 million (the area of the regional transfusion centre). An audit was undertaken in 2000 analysing use of plasma products and was published: discrepancies in use were identified between hospitals and the following represents a repeat analysis over the interim 5-year period.

A questionnaire was distributed to 15 hospitals with an 87% response rate. This compared April 2004 to March 2005 with the same period 1999–2000.

The results showed a decline in blood product use during this period despite a 2.9% increase in bed numbers within the region. Since 2000, there was a 9.1% reduction in red cell transfusions, but an even greater reduction in FFP (17%) and cryoprecipitate (20%) used. Using bed numbers as a surrogate reflection of activity, a mean of 13.65 red cell units were issued per bed (range 7.68–21.32, SD 4.51) in 2000, compared to 11.99 in 2005 (range 5.15–17.91, SD 3.34)  $P = 0.046$ .

Repeat analysis identified a substantial change in practice in previously outlying hospitals in terms of blood product use, with resultant savings of around £250 000 per annum.

A marked difference in blood product use between the two cardiac surgery centres was observed, which was thought to be attributable to regular use of thromboelastography in one.

Interestingly, 15% of centres still continue to take Rhesus D status into consideration on FFP issue whilst 23% consider it in females of child-bearing age, contrary to recent guidance from the BCSH.

It was also observed that no standard laboratory procedures were in place for the issue of methylene blue treated FFP and only 50% of the hospitals with potential use of this product had it in stock.

The report demonstrates that effective audit can improve and change clinical practice – something that is often questioned.

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# **A multi-centre prospective observational study of platelet transfusion practice in neonates with severe thrombocytopenia**

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Platelet transfusion practice in neonates is not evidence-based and there is a lack of data relating transfusion to clinical outcome. Neonates with platelets  $<60 \times 10^9/l$  were studied prospectively at 7

NICUs Mar 05–Sept 06. Daily data were collected on minor (scored 0–7) and major bleeding. Blood counts and reason for transfusion and its effect on bleeding were prospectively recorded. At the time of first analysis, 145 of 167 (87%) eligible neonates were enrolled (85% <37 weeks GA, 61% male). There were 186 episodes of thrombocytopenia (1606 study days) and 309 platelet transfusions given to 91 (63%) neonates. Fourteen per cent had major IVH (Grade 3/4) at study entry. Platelets ( $\times 10^9/l$ ) at study entry and at platelet nadir, and duration of count  $<60 \times 10^9/l$  were: 44 (33–54); 31 (19–42); 2 (1–5) days respectively. In transfused neonates, platelets ( $\times 10^9/l$ ) pre- and post-transfusion, and number of transfusions were: 27 (19–36); 84 (46–138); 2 (1–4) respectively. At least one episode of minor bleeding was recorded on 622/1606 (39%) study days, and scores recorded for 12 hours pre- and post each transfusion were (median

[IQR]): 1 (0–2) and 0 (0–1) respectively. New or extension of IVH bleeding to Grade 3/4 occurred in seven neonates and other major haemorrhage in 11 neonates (four pulmonary, four GI). 14% neonates died, 13 with major IVH bleeding (of which 10 had Grade 3/4 IVH at study entry); all received platelet transfusions (total 87; median 3 [2–7]). In summary, most thrombocytopenia episodes develop after 72 hours of life (median 5 days) and are of short duration (median 2 days). There is a clear distinction between the majority of thrombocytopenic neonates who receive one or two transfusions as prophylaxis with a good outcome, and the minority who suffer adverse outcomes including major haemorrhage (11%) despite transfusion. These data will be invaluable for planning definitive trials necessary for rationalising neonatal platelet transfusion.

## Poster Presentations: Transplantation

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### PBSC mobilisation with G-CSF alone as opposed to mobilisation with chemotherapy and G-CSF produces higher lymphocyte yields followed by faster lymphocyte recovery after high dose melphalan chemotherapy and autologous haemopoietic rescue in myeloma patients

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In myeloma patients, rapid lymphocyte recovery after high-dose chemotherapy and autologous haemopoietic rescue has been shown to be associated with better disease-free and overall survival. Here we report on a retrospective analysis of the dose of reinfused lymphocytes in the 30 consecutive myeloma patients who underwent consolidation chemotherapy with melphalan 200 mg/m<sup>2</sup> followed by a PBSC autograft in our department from March 2004 to March 2006. Whether patients were mobilised with G-CSF alone (10 patients) or intermediate dose cyclophosphamide and G-CSF was at the discretion of the treating haematologist. The dose of reinfused lymphocytes was determined with a single platform flow cytometry technique using a CD45<sup>high</sup>/SS<sup>low</sup> gate that was validated by comparison with the lymphocyte counts obtained on a Sysmex XE2100 haematology analyser in a separate batch of 17 peripheral blood samples with lymphocyte counts between 0.28 and 44.4  $\times 10^9/l$  ( $r = 0.9926$ ). For each patient, the PBSC graft size was chosen such that the CD34<sup>+</sup> dose was at least  $2.0 \times 10^6/kg$ . This resulted in a median lymphocyte dose in the autografts of 106.7 (range 24.9 to 455.5)  $\times 10^6/kg$ . Higher lymphocyte doses were significantly associated with faster lymphocyte recovery to above  $0.5 \times 10^9/l$  (Cox proportional hazards regression,  $P = 0.0245$ ). Patients mobilised with G-CSF alone received a graft with a higher lymphocyte content than those that were mobilised with chemotherapy and G-CSF (Mann-Whitney  $U$ -test  $P = 0.0062$ ). We conclude that the lymphocyte content can easily be incorporated in the routine quality assessment of PBSC harvests and that mobilisation with G-CSF alone favours rapid lymphocyte recovery after high dose melphalan chemotherapy and autografting.

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### Fludarabine may enhance the efficacy of DLI post HSCT for increasing mixed chimerism

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The increasing use of donor lymphocyte infusion to improve increasing mixed chimerism or minimal residual disease post transplant is well documented. In murine models, addition of fludarabine to DLI enhances donor chimerism and reduces the GVHD potential of through effects on a CD4plus CD44 (low) lymphocyte population. We report seven cases in which DLI was given with fludarabine for i-MC to enhance its effect.

Two females and five males with mean age of 4 years (range 6 months to 9 years), haematological diagnoses of thalassemia major (3), osteopetrosis (2), glycogen storage disorder (1) and JMML (1) were given a range of standard conditioning regimens. Peripheral blood stem cells were collected from haploidentical (5), unrelated (1) and sibling donors. The mean CD34 cell dose was  $5.96 \times 10^8/kg$  (range  $1.18$ – $10 \times 10^8/kg$ ). Cyclosporin A was given as GvHD prophylaxis. Neutrophil engraftment occurred 12 days (range 1–21 days) and platelet engraftment 34 days (range 15–86 days) post-transplant. All patients received DLI for i-MC after a mean 102 days post-transplant with a mean donor chimerism of 67 (range 25–83). All had one dose of intravenous fludarabine 25 mg/m<sup>2</sup> as further immunosuppression prior to their second or subsequent DLI. The second DLI was after a mean 153 days post-transplant. Only three received a third infusion.

In six cases (86%) stable mixed or full donor chimerism was achieved. All patients with thalassemia major and osteopetrosis achieved >90% chimerism. The patient with GSD achieved stable mixed chimerism. The patient with JMML achieved full autologous reconstitution with no evidence of disease. Two had grade one skin GvHD, one self-limiting graft aplasia but none had CMV reactivation, severe infection or death.

This is the first report in literature of the use of fludarabine to enhance the effect of DLI in clinical cases. We have demonstrated that the correction of i-MC with fludarabine and DLI can be achieved without significant morbidity.

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### Donor lymphocyte infusion is an effective management of increasing mixed chimerism and high risk disease with minimal side effects

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The use of donor lymphocyte infusion has demonstrated its use in increasing mixed chimerism or minimal residual disease post transplant. Little data is available on the efficacy of this therapy in children. We retrospectively analysed 31 patients who received DLI at Birmingham Children's Hospital from 1998 to 2006 with a mean age of 5 years; fifteen female and sixteen male. Seventeen had malignant and 14 non-malignant conditions. Nineteen haploidentical, eight unrelated and four sibling transplants with mean CD34 cell dose  $14 \times 10^6/\text{kg}$  for haploidentical and  $5.75 \times 10^6/\text{kg}$  sibling and unrelated transplants.

Indications for DLI in the malignant cohort included (a) relapse (7) (b) locally determined pre-transplant high-risk features (6), (c) i-MC (4) (d) MRD (2). Two patients relapsed a year post initial treatment and were retreated with DLI. Indications for DLI in the non-malignant cohort (a) i-MC (12), (b) relapse of AA (1) (c) infection post-transplant (1).

Twelve out of sixteen had complete donor chimerism post DLI for i-MC. Four failed to show improvement in chimerism, 2/4 had non malignant diseases and have stable mixed chimerism and 2/4 had JMML had full autologous reconstitution with normal haemopoiesis. Twenty-seven out of thirty-one had Grade 1–2 skin GVHD or no GVHD. Only 4/31 patients had severe morbidity due to Grade 3–4 GVHD and all responded to conventional treatment. There were no cases with CMV reactivation. 2/31 had graft aplasia from which he recovered after temporary cessation of DLI. There were nine deaths. Six patients died from relapsed disease. One patient given DLI for i-MC, died of streptococcal pneumonia due to non-compliance with penicillin V.

Our experience demonstrates that DLI can be delivered safely in high risk malignant disease post transplant in children but further work is needed to define efficacy. The correction of i-MC with DLI can be achieved with minimal morbidity and mortality and to date 22/31 of the group survive disease free.

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### Neutrophil engraftment, septic episodes and length of admission are equivalent in patients who receive pegfilgrastim after PBSCT compared to those who receive daily G-CSF

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Patients with lymphoma who receive high dose BEAM chemotherapy followed by autologous peripheral blood stem cell transplantation have routinely received daily G-CSF on day + 6 following stem cell re-infusion to hasten neutrophil engraftment. Pegfilgrastim is a pegylated form of G-CSF which is released slowly into the circulation following a single subcutaneous injection. Studies in patients who have received Pegfilgrastim following chemotherapy in an attempt to reduce length of neutropenia have shown that 1 dose of Pegfilgrastim is equivalent to 11 doses of daily G-CSF. We have recently substituted 6 mg Pegfilgrastim administered subcutaneously on day + 6 instead of daily G-CSF. In this study we have compared the median duration of neutropenia, number of septic episodes and length of admission

between 16 consecutive patients undergoing PBSCT who received Pegfilgrastim and 21 consecutive patients who had received daily G-CSF. The median duration of grade 4 neutropenia (neutrophils  $<0.5$ ) was 8 days in both groups (range: daily G-CSF 6–24d; Pegfilgrastim 6–14). The number of septic episodes and severity was no different (one ITU admission in daily G-CSF group, none for Pegfilgrastim group). In addition the median length of hospital stay was 22 days in both groups (range: daily G-CSF 16–56 day; Pegfilgrastim 18–35). The daily G-CSF group had 3 patients requiring further doses of G-CSF as outpatients; none in the Pegfilgrastim group required any further G-CSF. In the daily G-CSF group, a median of 6 days (range 4–22) G-CSF was given to achieve a neutrophil count of  $>0.5$  for two consecutive days. It thus appears that a single dose of Pegfilgrastim administered on day + 6 following BEAM PBSCT is as effective as daily G-CSF. Decisions regarding the preferred formulation in this setting will need to take patient convenience and cost into consideration.

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### Engraftment of donor langerhans cells following allogeneic stem cell transplantation with reduced intensity conditioning (RIC) is delayed compared to other myeloid lineages

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Langerhans cells (LCs) are antigen presenting cells found in the epidermis. They are thought to originate from bone marrow precursors although studies in mice suggest they have the ability to renew *in situ*. Murine models have also shown that recipient LCs are required for development of acute graft-versus-host disease (GVHD) following allogeneic haemopoietic stem cell transplantation (allo-HSCT). Engraftment of LCs following human allo-HSCT has previously been assessed using migration from isolated epidermal sheets, which may be inaccurate because of differential migration capacity of donor and recipient cells. We therefore studied LC engraftment using fluorescence immunophenotyping and simultaneous in-situ hybridisation for X/Y chromosomes in sex mismatched transplants. Skin biopsies were performed at days 28, 56 and 100, 6 months and 1 year post-transplant on eight patients receiving alemtuzumab based RIC allo-HSCT regimens. Ten micrometer cryosections were taken, LCs labelled with anti-CD1a and X/Y chromosomes detected with the CEP X/Y probe kit. Slides were examined on a Zeiss LSM510 Meta confocal microscope and Z-stack images were collected. Chimerism of purified CD15+ and CD3+ peripheral blood cells was determined in parallel. Results are tabulated below and show that compared to CD15+ myeloid blood cells, donor LC engraftment is markedly delayed (days 28 and 56 paired *t*-tests give  $P < 0.0001$  and  $P = 0.0008$ , respectively). Of the eight patients studied, three developed biopsy proven GVHD and in all cases, recipient LCs were still present. These results show that LC engraftment after RIC-allo-HSCT is markedly delayed with persistence of recipient cells at up to 1 year.

Cell Type	Day 28	Day 56	Day 100	6 Months	1 Year
LC	24.6 (n = 8)	70.5 (n = 7)	86 (n = 7)	92.5 (n = 6)	97.1 (n = 3)
CD3+ PB	92.2 (n = 8)	87.3 (n = 6)	72 (n = 6)	83.2 (n = 4)	94.6 (n = 3)
CD15+ PB	100 (n = 8)	99.7 (n = 6)	92.5 (n = 6)	100 (n = 4)	100 (n = 2)

% donor chimerism at specific time points following allo-HSCT.

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## Free Communications: Haematological Malignancy

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**Induction therapy with high-dose dexamethasone alone in newly diagnosed myeloma patients produces a high partial response rate which is predictive of progression free and overall survival****DA Irvine, MJ Barnett, R Brinkman, DL Forrest, DE Hogge, J Lavoie, L Lei, SH Nantel, TJ Nevill, J Nitta, J Shepherd, K Song, CA Smith, C Toze and HJ Sutherland***Division of Haematology, Leukaemia/BMT Program of BC, Vancouver General Hospital, BC Cancer Agency and University of British Columbia, Vancouver, Canada*

Induction followed by autologous stem cell transplant (ASCT) is standard treatment for eligible myeloma patients. The strategy in Vancouver has been to use single agent Dexamethasone as primary induction therapy. Between January 1998 and June 2005, 406 patients were referred for treatment of myeloma. We retrospectively reviewed all patients to determine rate and depth of response to Dexamethasone and to determine if response predicted survival. One hundred and twenty-four patients were ineligible due to prior therapy or no treatment. Analysis was performed on 282 patients. All received single agent Dexamethasone as primary therapy given at 40 mg days 1–4, 9–12, 17–20 every 28 days for at least two cycles. Response to Dexamethasone was as follows; nCR (electrophoresis negative) 4%, PR1 (>90% reduction in paraprotein/involved Ig subtype) 10%, PR2 (>50% reduction) 43%, MR (<50% reduction) 20%, NR (within 25% of baseline) 14% and progression (increase by >25%) 7%. Two hundred and twelve patients had ASCT with a melphalan containing regimen following stem cell mobilisation with cyclophosphamide and GCSF. Twenty-nine had myeloablative allogeneic transplantation. Mean follow-up was 35.5 months. In univariate analysis; OS was significantly influenced by ISS score ( $P < 0.001$ ), beta2microglobulin  $\geq 3.5$  mg/l ( $P < 0.001$ ), Hgb  $\geq 10$  g/l ( $P = 0.006$ ) and Ig subtype ( $P = 0.024$ ) while PFS was affected by beta2microglobulin  $\geq 3.5$  mg/l ( $P < 0.001$ ), Hgb  $\geq 10$  g/l ( $P = 0.006$ ) and depth of response to Dexamethasone ( $P < 0.001$ ). Achieving MR or better significantly influenced median OS ( $P = 0.018$ ) and PFS ( $P = 0.001$ ). Multivariate analysis confirmed depth of response significantly affected PFS ( $P = 0.008$ ). In conclusion, this data suggests that initial response to Dexamethasone is an important prognostic factor in patients with myeloma. Whether this relates to reduction of tumour load or because responsive disease is suggestive of lower risk tumour biology is unknown however it seems rational to evaluate new therapies in an attempt to improve depth and frequency of initial response and assess whether this influences long-term PFS.

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**The 'MILE' study achieves >95% prediction accuracy using gene expression profiling for the diagnosis and sub-classification of leukaemia****KI Mills<sup>\*,†</sup>, A Kohlmann<sup>‡</sup>, M Williams<sup>‡</sup>, L Wiecek<sup>‡</sup> and T Haferlach<sup>†,§</sup>**<sup>\*</sup>Department of Haematology, Cardiff University, Cardiff, UK,<sup>†</sup>MILE Study Group on behalf of WP13 European LeukemiaNET (ELN), <sup>‡</sup>Roche Molecular Systems, Pleasanton, CA, USA, <sup>§</sup>Munich Leukaemia Laboratory (MLL), Munich, Germany

The MILE (Microarray Innovations in Leukaemia) study was initiated in 2005 to assess and compare the clinical accuracy of gene

expression profiling with current routine diagnostic workup using standardised protocols. Here, data is presented from MILE stage I where  $n = 1889$  retrospective samples were profiled in 11 individual laboratories using HG-U133 Plus 2.0 microarrays. In 98.2%, the generated gene expression profiles passed strict quality acceptance criteria. These profiles were combined with previous microarray data from Munich and Memphis to generate a dataset of 2916 patient samples within 16 subclasses of acute and chronic leukaemia (mature B-ALL with  $t(8;14)$ , Pro-B-ALL with  $t(11q23)/MLL$ , c-ALL/Pre-B-ALL with  $t(9;22)$ , T-ALL, ALL with  $t(12;21)$ , ALL with  $t(1;19)$ , ALL with hyperdiploid karyotype, c-ALL/Pre-B-ALL without  $t(9;22)$ , AML with  $t(8;21)$ , AML with  $t(15;17)$ , AML with  $inv(16)/t(16;16)$ , AML with  $t(11q23)/MLL$ , AML with normal karyotype or other abnormalities, AML complex aberrant karyotype, CML, CLL), MDS, as well as non-leukaemia and healthy bone marrow as control group.

A linear discriminant classification algorithm was developed with 91.4% prediction accuracy for the 18 classes, and in addition, highlighting genetic relationships between disease sub-classes. Mis-calls were mainly between MDS and AML with normal karyotype, therefore, a separate classification model was generated based on 2647 samples representing 17 classes (excluding MDS) which increased the prediction accuracy to 95.4% with nine classes achieving >97.2% accuracy. Based on these results a customized microarray has been designed and manufactured using 1449 probe sets for leukaemia classification.

In conclusion, the international multi-centre MILE study research program has demonstrated a very high accuracy of leukaemia diagnosis and classification using gene expression profiling and has built the foundation for an innovative customized microarray designed for a routine diagnostic application of microarray technology. The custom AmpliChip Leukemia is currently being prospectively analysed on an additional 2000 samples in Stage II of the MILE Study.

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**Investigation of *HOXA6* as a candidate gene in AML****GJ McGonigle, DPJ Finnegan, MF McMullin, TRJ Lappin and A Thompson***Haematology Research Group, CCRCB, Queen's University Belfast, UK*

Molecular profiling in AML has identified several candidate genes that may define prognosis and response to therapy, including the Class I homeobox gene *HOXA9*. The *HOX* gene network encodes master regulators of haemopoiesis. To quantify its contribution in AML, specific RQ-PCR analysis was performed on twenty-four *de novo* patient samples using a subset of genes (12 *HOX* and *MEIS1*) selected due to their recently reported expression in AML. *HOXA6* was the most highly expressed gene, substantially higher than *HOXA9*. Furthermore *HOXA6* was highly expressed in CD34<sup>+</sup>-enriched primary progenitors. Parallel studies with murine progenitors (c-Kit<sup>+</sup>, Lin<sup>-</sup>) and cell lines also showed a preponderance of *Hoxa6* expression over other family members. *Hoxa6* regulation following differentiation or growth factor stimuli was subsequently investigated in haemopoietic cell lines. *Hoxa6* expression decreased with cell differentiation in EML and FDCP-Mix A4 cells and growth factor depletion/replenishment studies in 32Dcl3 and Ba/F3 cells indicated cell-cycle regulation of *Hoxa6*. Direct evaluation of cell-cycle status, using Hoechst 33342 staining and cell sorting, identified

peak expression of *Hoxa6* during S-phase. We overexpressed *HOXA6* in Ba/F3 cells to gain functional insights. Ba/F3-A6 cells were examined on the basis of proliferation, maturation, cell-cycle status, growth factor-dependence and apoptosis. Ba/F3-A6 cells displayed a growth advantage over control cells in the presence of IL-3 and maturation was not impaired. Cell-cycle analysis showed a reduced number of cells in both G2M and S-phase, associated with accumulation in the pre G1-phase, indicative of increased apoptosis. IL-3 depletion studies of Ba/F3-A6 cells indicated substantial factor-independent growth compared to controls, implying oncogenic potential for *HOXA6*. A recent report demonstrated *Hoxa6* as a potential collaborator in a *Meis1*-induced murine model of AML. Together these findings suggest *Hoxa6* has the capacity to alter growth and survival of haemopoietic cells and identify *Hoxa6* as a novel candidate gene in AML.

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# Use of quantitative PCR approaches to predict relapse and direct molecularly targeted therapy with arsenic trioxide (ATO) in acute promyelocytic leukaemia (APL)

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There is increasing emphasis upon de-intensification of treatment and delivery of pre-emptive therapy for molecular relapse to improve management of APL, accompanied by greater use of ATO in newly diagnosed and relapsed disease. However, the optimal ATO dosing schedule remains unclear; moreover, such strategies depend upon the development of optimised protocols for minimal residual disease (MRD) detection using real-time quantitative PCR (RQ-PCR) that can be applied to all patients. We characterised translocation breakpoints in diagnostic samples from 144 consecutive cases of APL, derived largely from the MRC AML15 trial. In 2% breakpoints were atypical, precluding use of standardised Europe Against Cancer RQ-PCR assays and necessitating the development of patient-specific assays. RQ-PCR revealed significant variation (3-log range) in the relative level of *PML-RARA* expression in APL blasts, impacting upon maximal achievable sensitivity for MRD detection (median 1 in  $10^{4.2}$ , range 1 in  $10^{2.3-5.8}$ ). Parallel detection of reciprocal *RARA-PML* transcripts improved assay sensitivity in 35% of patients and enhanced detection of MRD in remission samples. In relapsing patients, the rate of increase in *PML-RARA* transcripts varied between cases (median 0.8 logs/month, range 0.2–1.9), with molecular conversion occurring in BM ahead of PB. Two ATO dosing regimens were evaluated in 32 patients failing ATRA + chemotherapy: the conventional regimen (A): 0.15 mg/kg/day ( $n = 14$ ) or an alternative regimen (B): 0.3 mg/kg/day  $\times$  5 day, then 0.25 mg/kg  $2 \times$  /week ( $n = 18$ ). ATO regimen B, which is easier to administer, was comparable to the conventional schedule in terms of toxicity profile and efficacy, inducing molecular remission in the majority of patients treated in molecular or frank relapse. Overall survival in ATO treated patients from time of relapse was 61% at 3 years, which compared favourably with the survival of patients relapsing following ATRA + chemotherapy in the MRC AML10 and 12 trials prior to the

availability of ATO (47% at 3 years). These findings carry implications for the use of ATO and optimal scheduling of MRD assessment to realise individualised molecularly directed therapy in APL.

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# Outcomes of fully haploidentical hematopoietic stem cell transplantation compared to unrelated cord blood transplantation in children with acute lymphoblastic leukaemia. A retrospective analysis on behalf of Eurocord, Pediatric disease and Acute Leukemia Working Party of EBMT

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Both haploidentical T-cell depleted HSCT (Haplo) and unrelated umbilical cord blood (UCB) are established alternative stem cell sources for allogeneic transplantation in the treatment of high-risk acute lymphoblastic leukaemia (ALL). We have compared the outcome of these two approaches by performing a retrospective comparison of paediatric patients with ALL (16 years or younger) receiving a Haplo ( $n = 118$ ) or UCBT ( $n = 341$ ) in EBMT-Eurocord centres between 1998 and 2004. There were no significant differences in white blood cell count at diagnosis, immunophenotype, remission status at transplant, history of prior autograft, use of TBI in the conditioning regimen and year of transplant between groups. However, haplo recipients tended to be older, had CMV positive serology and  $t(9;22)$  more frequently. Unadjusted outcomes after a median follow-up of 56 months (range 1.3–115) and 24 months (range 2–96) for Haplo-PBSCT and UCBT patients respectively are summarized in Table 1.

Failure of engraftment was significantly higher following UCBT 23% compared to 11% in Haplo recipients ( $P = 0.007$ ). In multivariate analysis adjusted for differences between the groups and prognostic factors, relapse incidence was higher in haplo recipients compared to UCBT (RR = 1.7,  $P = 0.01$ ), but TRM and LFS were not significantly different. In conclusion, compared to Haplo, UCBT is associated with inferior engraftment, a higher incidence of grades II–IV acute GVHD, lower incidence of relapse but not different in terms of TRM and LFS, in paediatric patients with ALL. Therefore, in the absence of an HLA identical donor to treat a high risk ALL, both strategies are alternative options.

Approach	% of grade II-IV aGVHD	3 year TRM	3 year relapse	3 year LFS
Haplo ( $n = 118$ )	21%	50 $\pm$ 6%	56 $\pm$ 6%	22 $\pm$ 4%
UCBT ( $n = 341$ )	39%	46 $\pm$ 3%	45 $\pm$ 4%	29 $\pm$ 3%
p value	0.001	0.87	0.04	0.23



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**A clinical trial to evaluate peptide immunisation in chronic myeloid leukaemia (EPIC)****JM Rojas, K Knight, LH Wang and RE Clark***Department of Haematology, University of Liverpool, Liverpool, UK*

Chronic myeloid leukaemia (CML) is characterised by the BCR-ABL oncoprotein. Vaccination of CML patients with peptides from this junctional region could elicit/augment immune responses to CML cells. In our EPIC study, the patient's entry requirements were: (1) first chronic phase of CML, (2) expression of e14a2 (b3a2) BCR-ABL transcript, and (3) prior treatment with imatinib daily (at least 400 mg) at a stable dose for at least 6 months. Each patient received intradermally a cocktail of 3 BCR-ABL peptides: a 9-mer spanning the e14a2 region, the same 9-mer linked to PADRE (a helper peptide), and a 13-mer consensus e14a2 junctional peptide linked to PADRE. Peptides were administered at either 100 (five patients), 300 (five patients), 600 (six patients), or 1000 micrograms (four patients) with

sargramostim on six occasions over 2 months. Immune responses to the vaccine were monitored by IFN-gamma and IL-5 ELISPOT assays. Molecular responses were assessed by quantitative real-time PCR of BCR-ABL mRNA. Detectable anti-PADRE CD4<sup>+</sup> T-cells responses in all 19 assessable patients showed that the vaccination protocol could stimulate T-cell responses. Post-vaccination, anti-BCR-ABL CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were detected in 14/19 and 11/19 patients respectively. Serial molecular responses were available for at least 15 months on 19 cases. In patients where both anti-BCR-ABL CD8<sup>+</sup> and CD4<sup>+</sup> cells were detected, the BCR-ABL transcript levels were reduced further than in patients showing only partial or no anti-BCR-ABL immune responses (median reduction from baseline: 31.4% vs 73.2%,  $P < 0.01$  Mann-Whitney two tailed). Interestingly, in 12/13 patients in at least major cytogenetic response at baseline, we observed at least a 1 log fall in BCR-ABL transcripts. Moreover, vaccination improved the fall of BCR-ABL transcripts in patients who had received imatinib for more than 12 months. These data suggest that BCR-ABL peptide vaccination may improve control of CML. Randomised studies are now required.

**Free Communications: BSHT Symposium**

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**ADAMTS 13 in non-thrombotic thrombocytopenic purpura conditions****M Scully\*, R Liesner†, C Burgess† and SJ Machin\****\*Haemostasis Research Unit, University College London, UK,**†Haemophilia Comprehensive Care Centre, Great Ormond Street Hospital for Children, London, UK*

ADAMTS 13 is the primary physiological modulator of the size of VWF in plasma and its association with thrombus formation in TTP, led to an investigation in non TTP patients. Normal range for ADAMTS 13 activity is 66–126%. Ninety two samples from children attending outpatients, median ADAMTS 13 activity was normal (84% (13–167%) in cases <12 months, 93% (27–154%) in 1–6-year-olds, 105% (0–201%) in 6–12-years-olds and 78% (50–121%) in children >12 years). In 26/92 cases, ADAMTS 13 was <66%; 10 cases with ADAMTS 13 activity <50% – three had a reduced platelet count (<150 × 10<sup>9</sup>/l). Median ADAMTS13 activity in, primarily children, with bleeding disorders pre- (91.1%, range 69.2–176.6%) and post-factor concentrate replacement (84.8% range 55.4–146.2%) and Haemolytic Uraemic Syndrome (HUS) cases (62.5% range 33–89%) were within the normal range. Forty-eight children admitted to intensive care unit (varying medical conditions), 64% had reduced ADAMTS 13 activity (median 45%, range 0–65%) with no significant difference in platelet counts (median 261 × 10<sup>9</sup>/l-ADAMTS 13 <66% vs 276 × 10<sup>9</sup>/l-ADAMTS 13 >66%). VWF:ag levels were raised (>150 IU/dl) in 28/38 cases (73%) and in those with VWF:ag levels <150 IU/dl, only 2/38 cases had ADAMTS 13 activity <66%, but in those cases with VWF:ag >150 IU/dl, 16/38 cases had ADAMTS 13 activity <66%. In 100 unselected adult inpatients samples, 35 had ADAMTS 13 activity below the normal range (<66%); 15/35 were ITU patients. In patients with activity <66%, VWF:ag was significantly higher than patients with normal ADAMTS 13 activity (297.55 IU/dl v's 197.8 IU/dl) and unrelated to platelet count (215 × 10<sup>9</sup>/l [39–512 × 10<sup>9</sup>/l] vs 233 × 10<sup>9</sup>/l [24–636 × 10<sup>9</sup>/l] respectively). Ten out of twenty with ADAMTS 13 <66% had IgG antibody levels >4.2%. There were 17 deaths: five had ADAMTS 13 <66%. Therefore, the inverse relationship between ADAMTS 13 and VWF may be important in

thrombosis/multi-organ dysfunction in patients with a prominent inflammatory component.

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**Tissue factor and tissue factor pathway inhibitor levels in coronary artery disease: correlation with the severity of atheromatosis****LM Lima\*, MO Sousa\*, LM Dusse\*, MC Lasmar\*, BA Lwaleed† and MG Carvalho\****\*Federal University of Minas Gerais, Brazil, †University of Southampton, UK*

Atherosclerosis and its subsequent thrombotic complications are a major cause of morbidity and mortality in the western world. Tissue factor (TF) may contribute to atherothrombosis. *In vivo* TF activity is restrained by a major physiological inhibitor known as tissue factor pathway inhibitor (TFPI). Here we determined plasma TF and total TFPI levels in subjects undergoing coronary angiography. The relationship between these and the severity of coronary artery disease (CAD) was also assessed. Using ELISA assays, plasma TF and total TFPI levels were measured in subjects with normal coronary arteries ( $n = 20$ ; controls), mild/moderate atheromatosis ( $n = 18$ ) and severe atheromatosis ( $n = 30$ ). In addition, plasma lipoprotein(a) and D-Dimer (D-D) were analysed using ELFA and turbidimetric assays. The extent of CAD was assessed using coronary angiography. The severe atheromatosis group showed significantly high TF levels compared to controls ( $P < 0.01$ ). Increased TF levels were associated with coronary stenosis of more than 70% of the luminal diameter. Plasma lipoprotein(a) levels were also significantly increased in subjects with severe atheromatosis compared to those with mild/moderate atheromatosis ( $P < 0.001$ ) or controls ( $P < 0.0001$ ). The difference in lipoprotein(a) levels between controls and mild/moderate atheromatosis group was also significant ( $P < 0.0001$ ). The presence of CAD associated with increased TF ( $r = 0.42$ ,  $P < 0.0001$ ) and raised lipoprotein(a) levels ( $r = 0.63$ ,  $P < 0.0001$ ). For all groups there was a positive association between TF and TFPI ( $r = 0.34$ ;  $P < 0.01$ ), TF and D-D ( $r = 0.28$ ,  $P < 0.05$ ) and TF and lipoprotein(a) ( $r = 0.33$ ,  $P < 0.01$ ). In conclusion, increased plasma TF

levels in subjects with coronary artery disease are associated with the severity of atheromatosis. We observed an association between increased plasma TF levels and coronary stenosis of more than 70% of the luminal diameter. The advanced atherosclerotic injuries, the fatty core of the disrupted plaque and the increase in the macrophages and smooth muscle cells may have resulted in the observed rise in plasma TF levels.

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### What do elevated D-dimer levels mean in patients without venous thrombosis (VTE)?

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Use of D-dimer levels with clinical probability scores in the diagnosis of VTE is well established. High quantitative D-dimer levels have recently been shown to be predictor for poor survival and underlying malignancy in patients with VTE. Do quantitative D-dimer levels in patients without VTE have similar predictive value?

This study included 2263 (F: 1518; M: 745) patient episodes from a database of patients without VTE at a teaching Hospital, between 2001 and 2005. All patients with suspected VTE underwent a Doppler ultrasound examination to rule out VTE. D-dimer assays were done using Bio-Merieux kit. Database was regularly updated using hospital information systems, questionnaires and clinical review. Statistical analysis was carried out using SPSS 13.0 for Windows software. Overall survival (OS) was estimated by the Kaplan-Meier method. Median age at presentation was 69 years. Median D-dimer level was 1000 µg FEU/ml. Fifty-two per cent patients had a D-dimer level of >1000 µg and 2% had a D-dimer level of >8000 µg. Sixty-five per cent patients were aged >60 years. Median follow up was 22 months. D-dimer level >1000 µg, >4000 µg and >8000 µg were associated with decreased OS (Log rank test: *P* value: 0.002, <0.001 and <0.001). Age >60 year was also associated with decreased OS (*P* value: <0.001). D-dimer >8000 µg and age >60 year were an independent poor prognostic factor for OS on Cox regression analysis (*P* value: <0.001). 27.5% patients with a D-dimer level >8000 µg had cancer (Fisher's exact test; *P* value: 0.003). Seventeen per cent patients with a D-dimer level >4000 µg had cancer (*P* value: 0.04). 12.4% of patients with a D-dimer level >1000 µg had cancer (*P* value: 0.02).

This study shows elevated D-dimer levels even in patients without VTE is a marker for poor survival and a predictor for underlying

malignancy. This suggests heightened fibrinolytic activity in the absence or presence of established venous thrombosis may be a marker for underlying malignancy and is associated with poor prognosis. Further studies are warranted to establish in different medical conditions the presence or absence of increased fibrinolysis and impact on clinical outcome.

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### Thrombin generation: a comparison of assays using platelet poor and platelet rich plasma and whole blood samples from healthy control subjects and patients with a history of venous thromboembolism

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The monitoring of thrombin generation (TG) using platelet poor (PPP) and/or platelet rich (PRP) has been proposed as a global assay to assess coagulability. These assays exclude other cellular components of whole blood (WB) that may significantly contribute to the haemostatic process. We have now developed a fluorogenic WB TG assay and have compared TG in PPP, PRP and WB samples from 30 healthy control subjects (HCS) and 49 patients with a history of venous thrombosis (VTE). The platelet count in PRP was adjusted to the actual platelet count of the subject/patient using autologous PPP. Reference ranges (5th to 95th percentile) were established for peak height (PH) and endogenous thrombin potential (ETP) using PPP (PH 253–361 nM, ETP 1116–1681 nM/min), PRP (PH 131–214 nM, ETP 1170 to 1990 nM/min) and WB (PH 160 to 304 nM, ETP 1405 to 2323 nM/min) in samples from the 30 HCS. The VTE group PH and ETP values were significantly higher than the healthy control group in the WB samples (*P* = 0.003 and 0.003, respectively). Raised PH and/or ETP values for PPP, PRP and WB were seen in eight, eleven and twenty-five patients respectively. Peak height and/or ETP values were not consistently raised between the sample types. Our results suggest that the WB TG assay may be more sensitive to increases in TG in patients with a history of VTE than the PPP and PRP TG assays.

## Free Communications: Nursing Symposium

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### Sickle cell care pathways – why should we use them?

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Sickle Cell disorder is the generic term for a group of inherited blood disorders affecting the red blood cells. There are estimated to be 12 500 sufferers throughout the UK. In the South Wales area the patient cohort is small with less than 100 affected people which, historically, meant that care of patients in crisis was variable. The BSH guidelines for management of these patients, issued in 2003,

were used as the basis for a care pathway for use with all patients in Crisis within UHW. the pathway was designed to be used by any member of staff, anywhere in the Trust, regardless of haematological knowledge. Whilst used effectively with the majority of Sickle Cell patients, the importance of the pathway was not fully appreciated until a 'critical incident' in 2005. This presentation gives an overview of the patient involved in the form of a case study. The treatment history will be outlined and the presenting problems described. The results of not using the care pathway will be discussed in detail along with questions raised by this case and implications for future practice.

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### **The effect of iron deficiency anaemia on red cell transfusion in colorectal patients**

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The purpose of this study was to evaluate the incidence of preoperative anaemia in patients presenting with colorectal disease, and the influence of the anaemia on transfusions in the peri-operative period. Over a 6-month period 121 patients presented at the hospital colorectal multi-disciplinary meeting (MDT) were identified. Thirty-nine of these patients were anaemic, and 24 received between 1–19 U of blood. Fourteen of these patients had indices suggestive of iron deficiency, and they received 59 of the 167 U of blood used by the patients in this study. The anaemia was diagnosed at a median of 28 days pre-operatively (range 2–120 days), and a median of 26 days prior to transfusion (range of 2–300 days). The use of parenteral iron might have prevented half of these transfusions, we are planning to start an early diagnosis and intravenous iron intervention pathway for anaemic patients presenting to the colorectal MDT.

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### **Developing a patient focused service for myeloproliferative disorders – the telephone follow-up clinic**

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**Aim:** Through service redesign improve the quality of care provided to patients with chronic haematological disease and create additional capacity for formal outpatient follow-up.

**Background:** Each year in the NHS there are 37 million 'follow-up' appointments. A significant proportion of these follow-up visits are clinically unnecessary, create inconvenience and anxiety for patients and waste valuable resources. Seventy-five per cent of all outpatient 'Did Not Attend' (DNA) are for follow-up appointments (DOH, 2004). There are more than four million follow-up DNAs per annum, which costs the NHS more than £100 million a year.

The nature of haematological disease means that some patients are followed up on a long term basis as outpatients at varying degrees of frequency ranging from monthly to annually. These patients often attend hospital for a review of current blood parameters and are then advised that their disease is stable and a further follow-up appointment is scheduled. This frequently means that the patient has to travel to the hospital, pay to park, wait for a blood test, wait for the test result and then wait to see the doctor for a very short consultation.

**Method:** The development of a Nurse Consultant post gave the opportunity to remodel services to improve the patient's experience. This paper gives account of the development and evaluation of a nurse led telephone follow up service for patients with stable haematological disease.

**Findings:** Responses to a patient satisfaction survey identified that overall the majority of patients found the system to be effective and convenient for them with 90% indicating they preferred to be telephoned. In the first year the telephone clinic has saved 300 outpatient department follow-up appointments.

**Conclusions:** From this experience it would appear that telephone follow-up positively impacts on the patient and the service. Patients receive appropriate and timely care in the right setting and this in turn has created additional capacity in the outpatient setting for those that require it.

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### **Annual consultant review of patients in nurse led clinics; what does it achieve?**

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We established a nurse led clinic for patients with myeloproliferative disorders (MPD) in 2004. Stable patients with MPD are reviewed by a haematology clinical nurse specialist (CNS) either in the outpatient clinic or by telephone. The patient's symptoms and blood count are reviewed and treatment (medication/venesection) adjusted according to agreed parameters. Medical advice is available from a consultant or specialist registrar (SpR) in an adjacent haematology clinic if the patient has new or changed symptoms or unexpected abnormalities in the blood count.

There are now 107 patients with MPD in the nurse led clinic, with an average of 14 patients attending weekly in 1.5 clinics. All patients have an annual clinic appointment with a consultant, or SpR to review their treatment and progress, and for clinical examination to assess liver and spleen size. We have prospectively audited this annual medical review.

To date, 22 patients have been reviewed, 11 by a consultant and 11 by an SpR. Treatment advice by the haematology CNS was deemed satisfactory in 100% of cases as was documentation in the patients' notes and letters to the patients' GP. Clinical examination of the patients revealed that no patients had splenomegaly, including 2 patients who had previously had a palpable spleen. All patients were satisfied with their treatment in the nurse led clinic and 94% could not suggest any changes to improve the service.

This audit demonstrates that patients can be safely managed by a suitably trained nurse over long time periods. Routine annual medical review does not alter patient management. We now intend only to review patients medically if a new problem arises. This will further reduce medical follow-up, allowing us to increase new patient capacity. This will facilitate the department meeting outpatient waiting time targets.

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### **Improving uptake of patient self-testing in the anticoagulation clinic**

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Several studies have demonstrated that the quality of oral anticoagulation (OAT) may be improved by patient self-testing (PST). However, these studies used only selected patients who are already established on warfarin, and the uptake was disappointing, at around 10–25% of those eligible, prompting suggestions that PST is desirable to only a minority of patients. The aim of this study was to determine whether patients would accept PST more readily if offered from the start of treatment, and also to assess the effectiveness of PST in these patients. Three hundred consecutive patients referred to our anticoagulation clinic were prospectively assessed for suitability for PST. Exclusion criteria included: previous OAT, short therapeutic duration (e.g. pre-cardioversion); known drug/alcohol abuse; atypical INR target ranges; language barriers; and physical/intellectual impairment. One hundred and eighty-two out of three hundred (61%) were suitable of whom 85/182 (47%) consented to PST. Six patients did not commence self-testing after giving consent. On their

first visit to clinic 23% of eligible patients were within their target therapeutic range, with no significant difference between those electing for PST and those who declined. To date, 69/182 (38% of those eligible) patients have started PST of whom five have subsequently withdrawn from the study. The median time between first clinic visit and commencing PST was 27 days. After 3 months of treatment, there was no significant difference in the median time in

the target therapeutic range between PST patients and those who declined PST, 72% and 67%, respectively. The uptake of PST in this group of patients was superior to that in previous studies performed in the UK. Our data suggest PST is acceptable to many patients new to anticoagulation and that they are able to achieve a quality of OAT which is comparable to that obtained by a specialist hospital anticoagulation clinic.

## Poster Presentations: Cellular and Molecular Biology

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### Evaluation of dHPLC in the mutation analysis of type 1 plasminogen deficiency

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Plasminogen (PLG) deficiency is a rare disorder of fibrinolysis. It may be associated with chronic inflammation of the conjunctiva and other mucosal surfaces due to the development of 'ligneous' fibrin-rich pseudomembranes. These clinical symptoms are associated with homozygosity or compound heterozygosity of mutations found through out the PLG gene.

We have introduced denaturing high-performance liquid chromatography (dHPLC) analysis for mutation screening in this disorder and evaluated its use in comparison with direct sequencing of control and patient samples.

These techniques were used to provide genetic analysis for a 42-year-old female patient with ligneous cervicitis, a rare complication of PLG deficiency. Her PLG residual activity was 24% (nr. 74–104%), and antigen was 1.44 mg/dl (nr. 6–25 mg/dl).

Oligonucleotide primers were designed for specific amplification the 19 exons of the PLG gene, avoiding co-amplification of highly homologous genes. Parallel sequence analysis was carried out on an ABI 3130 genetic analyser with/without pre-screening using a Transgenomic WAVE. The patient was found to be a compound-heterozygote for Lys19Glu (the most common genetic defect associated with PLG deficiency) and a previously unreported mutation at the PLG exon 10 donor splice site (c.1256 +1 G>A); predicted to lead to a type 1 deficiency due to defective splicing. Additional polymorphic changes detected in control and patient samples included Asn91Asn, Cys238Cys, Phe295Phe, Gln342Gln, Asp453Asn and Gly743Gly. Eight further intronic polymorphisms were detected. dHPLC detected all changes that were found by sequencing, although some WAVE pattern changes were subtle. dHPLC was confirmed as a highly sensitive, rapid and economical technique for screening mutations. However, the highly polymorphic nature of the PLG gene should be taken into consideration when dHPLC is used during the genetic analysis of PLG deficiency.

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### Bone marrow microvessel density in chronic lymphocytic leukaemia: association with prognostic factors

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Angiogenesis is considered a prognostic factor in chronic lymphocytic leukaemia (CLL). However, there are insufficient data regarding

relationship of bone marrow angiogenesis to prognostic markers in CLL. Therefore, we assessed microvessel density (MVD) in bone marrow biopsy specimens from 22 untreated patients with CLL and 17 control biopsies. CLL cohort was further divided into subgroups according to clinical course (stable,  $n = 11$  vs progressive,  $n = 11$ ), Rai stage (0,  $n = 9$  vs I–IV,  $n = 13$ ), pattern of marrow infiltration (non-diffuse,  $n = 11$  vs diffuse,  $n = 11$ ), genetic abnormalities (favourable,  $n = 10$  vs unfavourable,  $n = 11$ ), and IgVH mutation status (mutated,  $n = 7$  vs unmutated,  $n = 14$ ). Neovascularization was assessed using immunohistochemical staining of endothelial cells with anti-CD34 monoclonal antibody and quantified using hot spot method. MVD was significantly elevated in CLL group in comparison to controls (mean  $\pm$  standard deviation [SD],  $75.6 \pm 50.6$ , 95% confidence interval [CI],  $53.2$ – $98.1/\text{mm}^2$  vs  $47.4 \pm 21.8$ , 95% CI,  $36.2$ – $58.6/\text{mm}^2$ ,  $P = 0.039$ ). However, there were no significant MVD differences between CLL subgroups with regard to prognostic factors. Interestingly, when each subgroup was compared to controls, only patients with diffuse bone marrow infiltration ( $P = 0.011$ ), Rai stage I–IV ( $P = 0.014$ ) and mutated IgVH genes ( $P = 0.019$ ) had significantly increased MVD. In conclusion, our study shows that microvessel density is significantly elevated in CLL. However, this difference is maintained diffuse pattern of infiltration, mutated IgVH genes and Rai stage I–IV only. We did not observe significant MVD differences between CLL subgroups with regard to classical or modern prognostic factors. Large prospective studies are necessary to confirm these findings and elucidate the real clinical relevance of bone marrow angiogenesis in CLL. Supported by grant NR/8373–3 and research project MZO 00179906 from Ministry of Health of Czech Republic.

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### The deltaN isoform of the TP73 gene is produced by cell cycle-dependent splicing during the G0–G1 transition in primary human T cells to regulate gene expression

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p73 is a member of the p53-family of transcription factors (p73, p53 and p63). A truncated form of p73 (deltaNp73) also exists that lacks the transcriptional activation domain and inhibits p53 as well as p73. However, deltaNp73 transcripts have not been reported in normal tissues or peripheral blood. We now show for the first time that deltaNp73 mRNA and protein are not present in quiescent (Go), primary human T cells but are induced post the Go–G1 cell cycle commitment point following stimulation with anti-CD3/CD28 or PMA/ionomycin. The deltaNp73 transcript could be produced either by activation of a promoter in intron 3 or from the 5' P1 promoter by alternative splicing. Bisulfite sequencing shows that the intron 3 promoter is hypermethylated in T cells throughout the cell cycle while the P1 promoter remains largely unmethylated. 5'-RACE results

with quiescent and stimulated T cells verify that *deltaNp73* transcripts originate from the P1 promoter. Additionally, RT-PCR analyses show that the transcript originating from the P1 promoter is present in Go and mature *deltaNp73* mRNA is produced by cell cycle-dependent splicing during the Go–G1 transition. We investigated the function of *deltaNp73* during Go–G1 by inhibiting its induction with two different siRNAs. Microarray analyses show that expression of the signal transduction proteins ERK1 and two during Go–G1 are dependent on *deltaNp73* and *deltaNp73* represses the expression of the pro-apoptotic protein PUMA, a known p73 target. We conclude that *deltaNp73* is produced in normal, human T cells during entry into the cell cycle and has a role in regulating normal gene expression. Moreover, the transcript originates at the P1 promoter and *deltaNp73* is produced by cell cycle-dependent splicing.

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### 'Excess' MCM proteins maintain genomic stability in human cells

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Normal DNA replication must be accurate and occur only once per cell cycle. Sites of DNA replication are specified by binding the origin recognition complex, which includes minichromosome maintenance (MCM) proteins. Paradoxically, in higher eukaryotes MCM proteins are present in 50–100-fold excess of that required for DNA replication. They are also downregulated by elevated expression of proteins such as cyclin E that occurs in cancers, including AML and breast cancer. We investigated why human cells need 'excess' MCM proteins and whether the reduction of MCM protein levels might contribute to a malignant phenotype. We determined the consequences of reducing the levels of MCM proteins in primary human T cells in which cell cycle controls and DNA damage responses are normal. Mass spectrometry sequencing of chromatin/nuclear matrix-bound proteins and western blotting identified that Mcm7 is not present in quiescent, normal primary human T cells but is induced in mid G1 after the Go–G1 commitment point. Reduction of Mcm7 with siRNA to <5% of normal during Go–G1–S-phase reduces chromatin-binding of each of the MCM proteins that form the DNA helicase. However, these cells still enter S-phase and replicate DNA, but exhibit DNA-damage responses involving activation of ATR and ATM pathways. Misrepair by non-homologous end joining (NHEJ) is also increased in cells depleted of Mcm7. However, cells depleted of Mcm7 do not undergo apoptosis, rather reducing MCM levels even

by 50% causes gross non-clonal chromosomal abnormalities normally found in genomic instability syndromes. M-FISH identified chromosome translocations, as well as loss and gain of individual chromosomes. Therefore, 'excess' MCM proteins that are present in a normal, proliferating cell are necessary for preventing DNA damage and for maintaining genome stability. Our data indicate also that genomic instability in cancers may result from a decrease in MCM protein expression.

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### Erythropoietin induces expression and phosphorylation of PI3-kinase p85alpha subunit in the lung carcinoma cell line H838

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Erythropoietin (Epo) is essential for the survival, proliferation and differentiation of erythroid progenitor cells. Binding of Epo to its receptor (EpoR) activates three major signalling pathways, namely JAK2/STAT5, PI3-kinase/Akt and MAPK. Recently EpoR has been shown to be expressed by malignant tissue. In contrast to erythroid cells the EpoR downstream signalling events in malignant cells are currently unclear. We have shown that the non-small cell lung carcinoma (NSCLC) cell line H838 expresses cell surface EpoR and that activation of the JAK2/STAT5, PI3-kinase/Akt and MAPK pathways occurs upon Epo binding. However, despite the evidence for downstream signalling in NSCLC cells, no increase in cellular proliferation was observed. To investigate the downstream signalling events in H838 cells, a microarray was performed to determine the effect of Epo stimulation at pharmacological levels. H838 cells were treated with 10 U/ml of Epo for 3 hours. As a result many genes were shown to have altered expression and statistical validation highlighted three, one of which, the PI3-kinase regulatory subunit p85alpha, was chosen for further study. This regulatory subunit is phosphorylated in response to Epo and plays a role in the prevention of apoptosis in erythroid cells. Real-time quantitative RT-PCR confirmed it was up-regulated 2 fold in H838 cells when treated with Epo for 3 hours. In contrast, the erythroleukaemia cell line, UT7, showed no up-regulation in the presence of Epo. At the protein level increased phosphorylation of the p85alpha subunit was observed during treatment with Epo at concentrations of 10 U/ml and 100 U/ml for between 30 min to 3 hours. Further studies are required to define the role that PI3-kinase p85alpha plays in Epo induced signalling in the NSCLC cell line H838 and whether it enhances cell survival in the absence of proliferation.

## Poster Presentations: General Haematology

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### Idiopathic erythrocytosis is associated with a risk of thrombosis - a retrospective case series review

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Idiopathic Erythrocytosis (IE) is a diagnosis given to patients who have an absolute erythrocytosis (red cell mass > 25% above mean

predicted value) without a known form of primary or secondary erythrocytosis. The natural history of IE is not well documented. We report results of a follow-up study of 80 patients (44 male and 36 female) diagnosed with IE from the UK and the Republic of Ireland over 10 years. The diagnosis was made on the basis of a raised red cell mass, absence of Polycythaemia Vera (PV), and the exclusion of secondary erythrocytosis. Older case studies of patients with 'IE' identified a more heterogeneous group of patients; more recent reviews suggest low rates of both transformation to myelofibrosis/acute leukaemia and thrombosis (1% patient-year). Average age at diagnosis was 34.5 (2–74 years). Erythropoietin levels were low in 18

(23%) and normal/high in 59 (74%). Fourteen patients had a family history of erythrocytosis. Mean follow up is 9.4 years (range 1–39). Twenty-four are no longer diagnosed with IE, eight have myeloproliferative disease; 13 a molecular abnormality likely to account for erythrocytosis (11 VHL, 1 PHD-2, 1 EPO-receptor mutations); and three secondary erythrocytosis. Fifty six patients are still classified as IE; 52/56 are alive (deaths: lung cancer, RTA, sepsis, unknown). Treatments of IE is venesection (35), hydroxyurea (3), none (11), unknown (2). Twenty take aspirin, 1 each warfarin/ dipyridamole and 30 neither. Four had thromboembolic complications (3 CVA/TIAs and 1 recurrent DVT) at/before diagnosis. Subsequently 9 thromboses occurred in eight patients – 7 arterial (1 CVA, 3 TIAs, 1 MI, 2 PVD); 2 venous (DVT/PE). The rate of thrombosis (1.6% patient-years) is lower than that in PV and is consistent with other case series. Molecular defects continue to be identified in this group and future investigation is likely to reveal further abnormalities.

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### Use of free light chain measurements as prediction of response to induction chemotherapy

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**Aim and Rationale:** Over 90% of multiple myeloma patients present with abnormal serum free light chain (FLC) concentrations. FLC have shorter serum half lives than intact immunoglobulins making them attractive candidates to monitor rapidly patient responses to chemotherapy. In this study, newly diagnosed patients had serum FLC and intact immunoglobulins monitored at frequent intervals during induction chemotherapy.

**Methods:** Serial serum samples were collected from 15 patients with newly diagnosed multiple myeloma. Where possible, samples were taken on treatment days 0, 4, 8, 21, 30, and then monthly to a maximum of three months. Patient characteristics were: age 60–81 (median 71), male to female ratio (2:1) and monoclonal immunoglobulin IgGκ(4), IgGγ(3), IgAκ(4), IgAγ(1), LCκ(2), LCγ(1). Three treatment regimes were used, namely vincristine, doxorubicin, dexamethasone (VAD)  $n = 5$ , cyclophosphamide thalidomide and dexamethasone (CTD)  $n = 5$  and melphalan and prednisone (M + P)  $n = 5$ . Clinical response after 3 months was assessed using the Blade criteria (Blade 1998).

Intact immunoglobulins and FLC were measured nephelometrically using a previously described immunoassay (Bradwell 2002). Serum FLC concentration was expressed as a percentage of the concentration at presentation.

**Results:** At the completion of the study, five out of fifteen patients had died, eight out of fifteen patients had minimal/partial response and two out of fifteen patients had complete/very good response. Assessment of individual patients' FLC concentrations at days 4 and 8 was not conclusive; however, any increase in concentration above presentation was indicative of a very poor clinical outcome. A good serum FLC response after 1 month (range 2–3% (mean 2.5%) of presenting concentration remaining) or a poor response (range 37–82% (mean 64%) remaining) corresponded with good and poor 3-month clinical outcomes respectively. The correlation between serum FLC response to chemotherapy and outcome could potentially allow the timely modification of ineffective chemotherapy regimes.

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### NT-ProBNP in AL amyloidosis: association with survival and changes after chemotherapy

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Cardiac involvement in AL amyloidosis is one of the most important determinants of survival and sensitive means of detecting such involvement remain limited. Cardiac natriuretic peptides are sensitive markers of cardiac dysfunction and are becoming a routine part of algorithms for diagnosis of heart failure. Serum NT-ProBNP is reported to be a promising biomarker of cardiac dysfunction and response to chemotherapy in AL amyloidosis. However, a number of patients in such studies had some abnormality of renal function, confounding interpretation. We studied NT-ProBNP serially in 169 patients with AL amyloidosis diagnosed from 1990–2005, in whom renal function was preserved. The median NT-ProBNP concentration was 49 pMol/l (healthy <35). Sixty-six points (41%) with cardiac involvement by echo had a median NT-ProBNP of 242 pMol/l vs 26 pMol/l for those without. NT-ProBNP <15 pMol/l was an independent marker of better prognosis, irrespective of septal thickness on echo and patients with such values had estimated 90% survival at 10 years. No evidence of cardiac involvement was found by any means in any patient with a NT-ProBNP less than 20 pMol/l, and in only one patient with a value less than 35 pMol/l. Following chemotherapy, only patients with a complete clonal response had a significant decrease in the median NT-ProBNP, with no significant change for those with a partial response and a rise for the non-responders. However, none of the patients with a decrease in NT-ProBNP showed echocardiographic improvement. NT-ProBNP is an independent marker for prognosis in AL amyloidosis but its behaviour and significance of changes after chemotherapy require more study.

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### A survey of haematology teaching in UK and Irish medical schools

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Following the publication of the first edition of Tomorrow's Doctors in 1993 by the General Medical Council (GMC) undergraduate medical education in the UK moved from a discipline based model of curriculum delivery to an integrated systems based approach. This transition has resulted in the loss of dedicated courses in a range of basic science and clinical subjects. The aim of this study is to explore the current status of teaching about haematology in UK and Irish Medical Schools.

In May 2006 a twenty item postal questionnaire was sent to 32 Medical Schools in the UK and five Medical Schools in the Republic of Ireland. Twenty one completed questionnaires were returned (response rate = 57%).

All of the respondents indicated that haematology is delivered as part of the core curriculum. Fourteen per cent of respondents offer Student Selected Components in haematology. Teaching about haematology is integrated throughout all years of the teaching programme in three Medical Schools. The majority of respondents indicated that teaching about this topic is delivered in years two and three. Ninety five per cent of respondents stated that students are

assessed in this subject. Fifty two per cent of respondents recommend a core text book in haematology while ninety five per cent reported making Study Guides available to students.

The results of this survey suggest that haematology teaching is integrated into the teaching programmes and is assessed in the Medical Schools that participated in the study.

All of the questionnaires returned were completed by academic haematologists. The 43% who did not respond may have minimal haematology input to the curriculum. The position of haematology teaching at undergraduate level will influence the doctors' knowledge and skills and recruitment into the specialty as teachers act as role models.

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# **High rate of renal recovery in patients with cast nephropathy treated by removal of free light chains using extended hemodialysis: a phase 1/2 clinical trial** **C Hutchison\*, M Cook\*, S Basu†, S Harding‡, G Mead‡, P Cockwell\* and A Bradwell§**

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Cast nephropathy from excess serum free light chains (FLCs) is the predominant cause of dialysis-dependent ARF in multiple myeloma. Only 12–20% of these patients recover renal function. Extended hemodialysis using a high cut-off protein-permeable dialyser (Gambro HCO 1100) for rapidly lowering sFLC was assessed for safety, efficacy and clinical outcomes in patients with multiple myeloma and dialysis-dependent ARF secondary to biopsy proven cast nephropathy.

Eight patients were studied: six new onsets; two with refractory/relapsing disease. The chemotherapy employed was dexamethasone and thalidomide for new disease; Velcade, doxorubicin and dexamethasone for refractory/relapsing disease. Extended dialysis (up to 12 hours/day) was very well tolerated. Consistent reductions in sFLC concentrations were achieved during each dialysis session (45–81%). Six patients, including the refractory/relapsing patients, achieved a sustained reduction in sFLCs of greater than 65% (range 65–95%). These patients subsequently became dialysis independent. Two patients did not respond to induction chemotherapy, had less sustained reduction in sFLC concentrations and remained on dialysis.

In conclusion, extended daily dialysis with a high cut-off dialyser rapidly reduced concentrations of sFLC in patients who were responsive to chemotherapy. Dialysis independence occurred in patients who achieved a >60% sustained reduction in sFLCs. Renal recovery occurred in 75% of patients compared with 10–20% in published comparative series of patients treated conventionally. This could have huge impact in the management and outcomes of patients with renal failure and multiple myeloma, if these results are replicated in more patients.

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# **A retrospective correlation study between V617F JAK2 mutation, aetiology of thrombocytosis and thrombo-embolic events**

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JAK2, a tyrosinase kinase gene, is located on exon 12. The substitution of valine to a phenylalanine (V617F) results in JAK2

mutation, which had been associated with myeloproliferative disorders. JAK2 mutation was recently found to be associated with higher haemoglobin level and leukocyte count leading to thrombotic complications (Wolanski *et al*, 2005). The objective of this study was to determine if any correlation exists between JAK2 mutation, aetiology of thrombocytosis, and thrombo-embolic events.

A retrospective study was conducted on all new and review patients with primary thrombocythaemia, polycythaemia rubra vera, myelofibrosis and reactive thrombocytosis attending the Haematology Department in Ulster hospital Dundonald.

A diagnosis of primary thrombocytosis was made in 22 (36.7%) patients. JAK2 was positive in 27.3% (6/22) patients. This incidence is low compared to previous published studies (Pargade *et al*, 2006). A history of thromboses was recorded in under a third of patients with primary thrombocythaemia (27.3%,  $n = 6/22$ ). Patients with primary thrombocythaemia and JAK2 mutation showed a 33.3% (2/6) chance of developing a thrombotic event, compared to 25% in the absence of JAK2 mutation.

A diagnosis of polycythaemia rubra vera was made in 14 (23.3%) patients. JAK2 mutation was present in 50% (5/14) patients (1 homozygous and six heterozygous). Thromboses occurred in 35.7% (5/14) of patients with polycythaemia rubra vera. Among patients with polycythaemia rubra vera as well as the JAK2 mutation, a 14.3% (1/7) chance of having a thrombotic event was observed; this risk was higher at 57% in the absence of JAK2 mutation.

In our study population, the incidence of JAK2 mutation in primary thrombocythaemia and polycythaemia rubra vera is lower than previously reported. The presence of JAK2 mutation appears to increase the risk of thrombo-embolic events among patients with primary thrombocythaemia; the risk was unchanged for myelofibrosis and reactive thrombocytosis. Conversely, in polycythaemia rubra vera, the risk of thrombosis was decreased in the presence of a JAK2 mutation.

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# **Presentation and survival of multiple myeloma: six years survey at Ulster Hospital**

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We retrospectively reviewed all patients diagnosed with Multiple Myeloma between 2000 to 2006 from our register. We were able to retrieve 60 out of 69 patient records. Out of 60 patients that were diagnosed with Multiple Myeloma 37 were identified as males and 23 as females (M:F = 1.6:1), and median age was 71.5 (36–89). Twenty-four (40%) patients were asymptomatic and were referred because of incidental finding of raised paraproteins. Symptoms included bone pain in thirteen (21.7%), anaemia in nine (15%), acute renal failure with anaemia three (5%), weight loss in three (5%), pancytopenia in two (3.3%) patients and hypercalcemia in one (1.7%) patient. Three (5%) patients progressed from previous MGUS. We also encountered rare presentation as amyloidosis in one (1.7%) patient, and cord compression in another one (1.7%). Majority of patients 48 (80%) were treated at presentation. Other patients were asymptomatic 12 (20%) and were observed. Patients were treated according to their age at presentation. Twenty-seven (45%) patients with age more than 70 years were treated with Melphalan with or without Prednisolone as a first line therapy. Patients with age less than 70 years were treated with VAD regimen four (6.7%) patients, ZDEX 10 (16.7%) patients, Cyclophosphamide one (1.7%) patient. Additional treatment such as Radiotherapy was given to 14 (23.4%) patients. Twenty-two (36.7%) patients needed bisphosphonates. Thalidomide was given to

7 (11.7%) patients. Eight (13.4%) patients were referred for stem cell transplant. Five (8.4%) patients had successful stem cell transplant done. All five patients are still alive and having regular follow up. Twenty-four (40.1%) deaths were documented. Survival range for these patients was 1 month – 45 months and mean survival age was 37.5 months. Sixty per cent of the patients diagnosed are still under regular follow-up.

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# Platelet alpha granule contents and bone marrow fibrosis in myeloproliferative disorders

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Platelet derived growth factors including transforming growth factor beta (TGFbeta) have been implicated in the pathogenesis of bone marrow fibrosis in myeloproliferative disorders (MPD). These growth factors are contained within platelet alpha granules along with beta thromboglobulin (betaTG). We measured plasma levels of TGFbeta and betaTG in 62 patients with MPD including five myelofibrosis (MF), 16 polycythaemia rubra vera (PRV), 38 primary thrombocythaemia (PT) and three MPD not otherwise specified, and compared them to 18 age matched controls. For patients, the degree of bone marrow fibrosis at diagnosis was obtained from pathology reports. The means and standard error of the mean (SEM) are shown in the table; statistically significant results ( $P < 0.05$ ) are denoted with an asterisk. Mean betaTG and TGFbeta levels were elevated in all MPD patients and each of the MF, PRV and PT groups compared to control ( $P < 0.01$  for all). Additionally, levels were elevated in MF and PRV compared to PT patients ( $P < 0.05$  for all). Diagnostic trephine reports were available for 52 patients; 46 with no, or mild to moderate reticulin fibrosis (group A/B) and six with fibrosis of grade 3 or greater (group C). Mean betaTG and TGFbeta were elevated in group C compared to Group A/B (262.0 vs 122.0 IU/ml,  $P < 0.01$  and 2959.2 vs 1777.4 pg/ml,  $P = 0.01$ ).

In conclusion, plasma levels of platelet alpha granule contents including TGFbeta are elevated in patients with myeloproliferative disorders, especially MF and PRV, and are associated with greater degrees of bone marrow fibrosis.

	Mean beta TG (IU/ml)	Mean TGF beta (pg/ml)
All MPD (62)	121.9* (14.2)	1813.4* (151.5)
MF (5)	246.6* (97.1)	3044.8* (1051.3)
PRV (16)	127.4* (16.8)	2015.2* (337.1)
PT (38)	83.0* (8.1)	150.3* (121.4)
Controls (18)	44.0 (3.8)	744.6 (58.8)

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# An audit of the use of recombinant human erythropoietin in a haematology department and its sequelae

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Cancer and cancer treatment-related anaemia (CRA) is a common clinical problem in the management of haematological malignancy.

The mainstay of treatment has historically been red cell transfusions. More recently, recombinant human erythropoietin (rHuEPO) is increasingly being used in this setting.

Clinicians today are faced with an expanding choice of erythropoietic proteins, and a wealth of data regarding appropriate dosing regimens, approved indications, efficacy and safety. The cost of rHuEPO is significant, and because not all patients respond to therapy, it is important that appropriate assessment of patients takes place pre-therapy, and that monitoring of response is rigid.

We undertook an audit of the use of rHuEPO in our department to look at these aspects. The findings showed that although red cell transfusions were avoided in approximately 60% cases of CRA, there was inappropriate prescription of rHuEPO for the following reasons: inappropriate trigger haemoglobin level, inadequate baseline checks, multiple dosing schedules, incorrect monitoring, inappropriate duration of therapy, and poor documentation of complications.

Critique of existing clinical guidelines led to the introduction of a departmental policy for rHuEPO prescribing encompassing:

1. Introduction of a specific rHuEPO prescription form which allowed pharmacy to decline issue of rHuEPO if appropriate baseline checks were not documented.
2. Review and amendment of the departmental standard operating procedure for the use of rHuEPO in CRA; recognition of functional iron deficiency as a cause of inadequate response.
3. Formulation of a departmental algorithm for the management of CRA.

As a result of these measures, there was significant reduction in departmental expenditure for rHuEPO equating to a saving of over £300 000 for the 6-month period following the introduction of the policy. This audit demonstrates how critical evaluation of departmental practice, with reference to relevant current guidelines, can lead to better clinical practice and significant financial savings.

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# Hyaluronic acid and liver function in myeloproliferative disorders

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Hepatic fibrosis has previously been described in haematological diseases, in particular the myeloproliferative disorders (MPD). Serum hyaluronic acid (HA) is used as a marker of hepatic fibrosis or cirrhosis in a number of liver diseases. We therefore assessed HA levels and standard liver function tests in patients with MPD. Sixty-two patients including, five myelofibrosis (MF), 16 polycythaemia rubra vera (PRV), 38 primary thrombocythaemia (PT) and three MPD not otherwise specified, were compared to 17 age matched controls. The mean results and standard errors of the mean (SEM), are shown in the table; statistically significant results ( $P \leq 0.05$ ) are denoted by an asterisk. HA was greater than the upper limit of normal (75 ng/ml) in 28 MPD patients (45%) and four controls (24%). The mean HA for all MPD was elevated compared to controls but this was not statistically significant ( $P = 0.19$ ). Subgroup analysis revealed greater mean HA levels in the MF and PRV patients compared to PT patients ( $P = 0.05$ ,  $0.02$ ). Mean bilirubin (Bili) and alanine aminotransferase (ALT) levels were similar in all patient groups. Mean alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) levels were elevated in MF and PRV compared to PT patients and controls (all  $P < 0.01$ ).



In conclusion, HA levels are commonly elevated in patients with MPD and are highest in patients with MF and PRV, who also have higher ALP and GGT levels compared to PT patients and controls.

	HA ( $<75$ ng/ml)	Bili ( $3-16$ $\mu$ g/l)	ALT ( $10-50$ U/l)	ALP ( $40-125$ U/l)	GGT ( $10-55$ U/l)
All MPD	78.5 (8.0)	9.9 (0.6)	26.5 (2.5)	97.0 (5.5)	57.0 (7.7)
MF	104.4* (32.2)	13.0 (2.5)	46.4 (23.7)	158.6* (41.8)	153.4* (65.2)
PRV	106.1* (23.2)	9.3 (1.1)	21.8 (2.4)	117.4* (8.9)	67.1* (11.7)
PT	61.9 (6.7)	10.1 (0.9)	25.9 (2.3)	76.5 (3.1)	36.5 (4.6)
Controls	63.6 (7.3)	8.2 (0.6)	30.7 (4.0)	86.6 (4.7)	38.5 (5.4)

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### Management of febrile neutropenia: a retrospective audit in a district general hospital setting

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Febrile neutropenia (FN) is one of the commonest reasons for admission following chemotherapy, needing prompt recognition and treatment. A retrospective audit was undertaken over 1-year of patients diagnosed with FN and subsequently admitted to the haematology ward. We assessed whether FN was correctly diagnosed, according to our local hospital protocol, by the admitting medical team and whether it was appropriately investigated and treated (correct antibiotics given within one hour and appropriate use of gentamicin).

Forty-three patient episodes were assessed. Thirteen (30%) did not meet the local criteria for the diagnosis of FN and six of these patients were treated as FN. Thirty (70%) had FN of which 23 (77%) were haematology patients, four (13%) oncology patients, and three (10%) had non malignant conditions with drug related neutropenia.

Of the patients who were diagnosed correctly with FN, 100% were investigated appropriately (blood and urine culture and chest x-ray) and received first line antibiotics (piperacillin/tazobactam or ceftazidime  $\pm$  gentamicin). However four patients (13.3%) had significant renal impairment and were given gentamicin without dose reduction. One patient did not receive gentamicin due to renal impairment, despite the protocol stating that a reduced dose should be given. This patient died as a result of overwhelming sepsis.

Only 17% received antibiotics within one hour according to the local protocol. In 43% there was no documentation about timing of antibiotics. Forty per cent received antibiotics within 1–5 hours. Of the patients who had FN, twenty-five were discharged home, four died of other causes and one died of sepsis.

This audit demonstrates a need for more accurate diagnosis of FN, more timely antibiotic administration and more cautious use of gentamicin in renal failure. These findings are useful for the education and training of front line clinical staff.

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### Audit of adult patients with neutropenia referred to general haematology outpatient clinic

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Isolated neutropenia of varying severity is a frequent reason for referral to general outpatient haematology clinics. Local experience indicated that such patients were rarely found to have a serious underlying diagnosis. We audited all referrals of patients with neutropenia to the general haematology outpatient clinic at Leeds General Infirmary over a 2-year period from January 2003 to 2005. The neutrophil count of all 477 new patient referrals and the notes of all 56 patients found to be neutropenic were reviewed. Ethnic minority groups were over-represented (15% were Afro-Caribbean, compared with 0.14% of the population). In 48 patients the final diagnosis was a benign (e.g. chronic idiopathic, ethnic or post viral neutropenia) or non-haematological one. A distinct group of patients with mild isolated neutropenia could be identified (defined as those individuals with neutrophils in the range of  $1-2 \times 10^9/l$  without other full blood count abnormalities). Seventy-five per cent of these patients were female, and their average age was 10 years less than that of the total group. No patient in this group was found to have a serious underlying diagnosis. Eight patients were diagnosed as having a haematological malignancy or aplastic anaemia but all had other abnormalities in the FBC. These data have been used to develop a policy for triaging referrals from general practice, with written advice being given rather than an outpatient appointment being made. It was recognised that a large proportion of such referrals could be prevented by appropriate advice being written on the full blood count report issued by the laboratory. In addition, it is important to recognise different reference ranges in ethnic minorities, especially in areas where they do not constitute a large part of the population. Adoption of these policies may help haematology services meet new government outpatient waiting time targets.

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### Survival and outcome of blastoid variant myeloma following treatment with DT-PACE

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Blastoid morphology in myeloma is a rare presenting feature associated with poor outcome, and hence effective treatment strategies need to be developed. Extramedullary myeloma also often has this morphology and is known to be associated with a poor outcome. Cells with these features often respond well to cell cycle active classic chemotherapy regimens and combination of these agents with thalidomide offers an interesting new approach, which may be useful in this setting. We have collected and analysed all such cases presenting in a 5-year period and evaluated their characteristics and outcome after treatment with DT-PACE  $\pm$  autograft. We analysed the results of 34 patients (median age 51.6 years, range 37–67) of whom 22 (60%) had extramedullary and/or blastoid type and 12 had relapsed/refractory disease. DT-PACE consists of dexamethasone 40 mg day 1–4, thalidomide 100–200 mg daily, and cisplatin 10 mg/m<sup>2</sup>/day, doxorubicin 10 mg/m<sup>2</sup>/day, cyclophosphamide 400 mg/m<sup>2</sup>/day, and etoposide 40 mg/m<sup>2</sup>/day all given on days

1–4, on a 28-day cycle aiming to deliver two cycles. Twenty patients received DT-PACE alone, and 14 DT-PACE followed by a melphalan autograft. The overall response rates (CR/PR) were 59% for the blastoid group and 27% for the relapsed/refractory group. Despite these initial good response patients with the blastoid variant had early disease progression (PFS 5 vs 13 months,  $P = 0.015$ ) and (OS 10 vs 13 months,  $P = 0.138$ ). Patients who tolerated the DT-PACE and were able to go on to the autograft had better PFS and OS when compared to the group who received the chemotherapy alone (PFS = 12 vs 4 months,  $P = 0.023$  and OS not reached vs 4 months,  $P = 0.006$ ). In conclusion we confirm the poor clinical outcome for this group of cases even with the intensive regimen DT-PACE, however, in contrast to previous treatment excellent response rates are obtained and some long-term survivors are found. This forms the basis against which future strategies aimed at improving this regimen utilising novel therapies can be compared.

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# **Report of the first case of CMV colitis as the presentation of multiple myeloma**

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Reactivation of CMV usually occurs in the immunocompromised patient due to defective T-cell responses. Reactivation has occurred in solid organ /bone marrow transplant recipients, hemodialysis patients on immunosuppressive drugs, and in HIV. CMV colitis is uncommon in patients who are not severely immunocompromised. Rarely it complicates inflammatory bowel disease- ulcerative colitis and Crohn's disease. CMV colitis as a presenting feature of multiple myeloma (MM) has not been previously documented. We report an 81 year old man who presented with diarrhoea and abdominal pain due to CMV colitis and was discovered to have multiple myeloma. The patient presented with 2-month right upper quadrant abdominal pain, diarrhoea and lower back pain. Abdominal and pelvic CT scans revealed segmental thickening of the sigmoid colon suggestive of colitis. Colonic biopsy confirmed CMV colitis with characteristic scattered enlarged cells staining positive with antibody to CMV. CMV PCR showed 6400 CMV DNA copies. Thoracolumbar X-rays and MRI scans demonstrated a crush fracture of D9 spinal region and cord compression with involvement of D7 and D8 vertebra. A soft tissue mass extended into the spinal canal. Biopsy of this revealed plasmacytoid infiltration. Serum electrophoresis revealed an IgG paraprotein of 20.7g/l with immunoparesis. Bone marrow showed 10% plasma cells with light chain restriction. Skeletal survey did not reveal lytic lesions. Bence-Jones protein levels were >95 per cent of total urine protein, beta-2 microglobulins of 6.93g/l, Ig G 26.8, IgA 0.44 (NR0.8-1) IgM 0.49 (NR 0.5-2) corrected calcium of 2.64 mmol/l, creatinine clearance 47 ml/min. HIV 1 and 2 and HTLV1 neg. Treatment with valganciclovir resulted in resolution of diarrhoea and abdominal pain and two separate negative serum PCR tests for CMV after treatment. Radiotherapy and cyclophosphamide, thalidomide and dexamethasone were initiated.

CMV colitis has not been reported previously in MM. Since T-cell dysfunction is known to be abnormal in MM patients CMV colitis though unusual, should be considered in clinically appropriate settings. CMV colitis when unexplained should have a serum electrophoresis performed as investigation of a possible underlying cause

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# **An audit of the economic consequences of early JAK2 testing in the investigation of a raised haemoglobin**

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The myeloproliferative disorders (Dameshek 1951), have until recently lacked a unifying pathophysiology. In 2005 a single acquired point mutation in the Janus Kinase 2 (JAK2) gene, at position V617F was reported. This gene is present in 95% of cases of polycythaemia vera (PV). Consequently several new diagnostic systems, which rely on early JAK2 testing, have been proposed (NEJM 2006; 355: 2452–66). Many centres include JAK2 testing without analysis of cost implications. We evaluated the health economic value of early JAK2 testing in patients with a raised haemoglobin and haematocrit. We audited all JAK2 requests generated by our hospital from 10.06.05 to 10.07.06 in patients with high haemoglobins (male Hb >17, Hct >0.5, female Hb >15, Hct >0.46) and correlated it with tests of serum erythropoietin (EPO), full blood count, urea, creatinine and blood volume studies. In 47 cases of a raised haemoglobin and haematocrit four JAK2 mutations occurred. In two of these, the EPO was below the lower limit of normal. Of 37 JAK2 negative cases, the EPO was below the lower limit of normal in five. In these five, no explanation for the low EPO levels could be found but, in one the haemoglobin normalised without treatment, another is under investigation for a renal mass, the third had a normocellular bone marrow and another had a hypocellular bone marrow with normal blood volume studies. We therefore do not believe these five cases are in the 5% of JAK2 negative PV. Our data suggest that JAK2 testing can be avoided if the serum EPO level is measured first. If testing is reserved for cases where EPO is <10 mU/ml a cost saving would be made and the same level of diagnostic precision maintained. In our study £3150 would have been saved. Further analysis of a larger number of JAK2 two positive cases would better identify the EPO level below which JAK2 testing should be performed. We are currently evaluating the use of a diagnostic algorithm based on these findings. (EPO-£39.90, Blood volume-£240, JAK2-£150).

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# **Hyperparathyroidism – associated polycythaemia: an analysis of 140 cases of hyperparathyroidism**

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There are several case reports of polycythaemia/erythrocytosis occurring in association with hyperparathyroidism, due to both parathyroid adenoma and carcinoma; the mechanism is unclear but in some cases the polycythaemia disappears on removal of the tumour. To determine the prevalence of erythrocytosis in hyperparathyroid patients we identified patients with high parathyroid hormone (PTH) levels attending our hospital between 2001 and 2006. We found 140 patients with elevated PTH and without end-stage renal failure. Full blood counts were available in 120 patients (85.6%). Thirteen (10.8%), including one male, had haemoglobin

and/or haematocrit values above the normal range. The haemoglobin values in the twelve females ranged from 14.4 to 18.3 g/dl (NR 11.4–15.0) and the haematocrit from 0.442–0.522 (NR 0.340–0.440), with two having haematocrit > 0.48; the one male had a haemoglobin of 16.9 g/dl (NR 13.5–17.0) and haematocrit 0.503 (0.39–0.50).

The subject with haemoglobin 18.3 g/dl and haematocrit 0.522 was a 54-year-old female referred for investigation of raised haemoglobin, who was hypercalcaemic with a persistently raised PTH level. Isotope studies showed a raised red cell mass with normal plasma volume and spleen pool, confirming a true polycythaemia. Her erythropoietin level was raised at 35.8 IU/l (NR 5.0–25.0), suggesting a secondary polycythaemia. She was negative for the JAK2 V617F mutation.

Erythrocytosis, of varying levels of severity, may occur in association with hyperparathyroidism and may be under-diagnosed. The case with high erythropoietin suggests that the erythrocytosis is likely to be secondary, however further work is needed to investigate the pathogenesis. We conclude that a calcium level should be included in the initial investigation of an erythrocytosis, and if the calcium is raised the PTH level should be measured. Conversely, a full blood count should be performed in the investigative work up of suspected primary hyperparathyroidism.

## Poster Presentations: Haemostasis and Thrombosis

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### A retrospective audit of a standard protocol for the rapid reversal of warfarin in patients with intracranial haemorrhage

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Intracranial hemorrhage (ICH) is a major complication of anticoagulation. The incidence is approximately 1% per year and mortality is about 60%. Risk factors include high INR, older age and cerebrovascular disease. Lower levels of consciousness, large bleed volume and haematoma expansion are associated with poor outcome. Early identification of ICH and prompt warfarin reversal are potentially critical in decreasing mortality and morbidity. To manage this complication the Northern Region Hematologists Group designed a protocol to rapidly reverse warfarin in patients with ICH. The protocol recommends administration of Vitamin K (5 mg IV) and a Prothrombin complex concentrate (PCC – Beriplex: 30 U/kg).

The protocol was assessed by retrospective audit. 26 patients (14 male; 12 female; median age 71 years (range 39–88) were identified with warfarin-associated ICH. Persistent headache (54%), decreased conscious level (38%) and confusion (35%) were the commonest presenting features. Hypertension (69%) was the commonest risk factor with INR > 4 in only 26%. Good compliance with the recommended protocol and rapid reversal of warfarin was demonstrated (Pre PCC administration: INR: median 2.6; range 1.5–10.15 min post PCC administration: INR median 1.1; range 1.0–1.4). The median time from hospital admission to vitamin K and PCC administration was 229 (range 100–460) and 323 (range 150–510) min respectively. Outcome (at 30 days): Independent (27%); moderate/severe disability (31%); death (42%).

This retrospective audit shows that a standard protocol for the reversal of warfarin in patients with ICH is effective and may be associated with improved outcome. However, in practice, the administration of both vitamin K and PCC does not occur rapidly following hospital admission. A prospective study would be required to assess whether more rapid reversal is associated with an improved outcome. Patient education is required to ensure persistent headache is reported as a significant number of patients were symptomatic for several days prior to presentation.

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### Clinical outcomes of point-of-care INR monitoring in primary care anticoagulation practice

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Approximately 500 000 people in the UK receive oral anticoagulant therapy (OAT), with this figure rising 10% per year and an increasing workload for laboratories. This workload, together with issues of patient autonomy and safety of care has resulted in the development of monitoring of the INR by point-of-care (POC) devices and one stop primary care clinics. Studies of POC INR testing show comparable performance to conventional laboratory testing, however, there is limited information regarding the clinical outcomes with POC monitoring.

To assess clinical outcomes of POC INR monitoring in primary care practice, a 12-month retrospective study was undertaken of nine GP practices with established anticoagulant clinics using POC monitoring. A postal questionnaire requested the total patient list size, numbers on warfarin, details of POC testing, the means of dosing and adverse events for thrombosis, bleeding and deaths.

Nine practices with 101 822 patients reported 1305 subjects on warfarin monitored by POC devices. All followed manufacturers' guidance on POC testing, undertook external quality control with the local Haematology laboratory, with warfarin dosing facilitated by computerised decisions support software (CDSS). There were three thrombotic and five haemorrhagic reported episodes with a respective incidence of 0.23 and 0.38 per 100 000 persons per annum, with no associated fatalities. Among surgeries reporting deaths, there were 26 cases amongst 337 warfarinised patients (7.7%), none due to haemorrhage or thrombosis.

Control of OAT by POC monitoring in primary care is safe and effective with a low incidence of recorded adverse events, comparable with patients monitored using conventional laboratory testing (O'Callaghan S, Laporte S, Camps F, Fuste N, Carrasco P, Tarres V. (1997) Hemorrhagic complications and Thrombotic Phenomena in Oral Anticoagulation of Elderly Patients. *Revista clínica española* 197, (5): 323–8.). Further prospective study should be undertaken, together with a conventionally monitored control group.

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# **The safety and efficacy of long term low molecular weight heparin (LMWH) in patients with portal hypertension**

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Portal vein thrombosis (PVT) is responsible for 5–10% of all cases of portal hypertension in Western countries. The association of PVT with an underlying pro-thrombotic disorder has been repeatedly reported and accounts for 10–12% of PVT cases in adults.

There are no prospective studies on the safety and effects of long term anticoagulation in cases of PVT where an associated pro-thrombotic history or defect exists. Paradoxically, lack of anticoagulation may accelerate GI haemorrhage from oesophageal varices secondary to further abdominal vessel thromboses.

A retrospective analysis of around 70 patients with PVT, where a pro-thrombotic state had been demonstrated, reported that these patients were likely to have recurrent thrombotic events in the portal venous system, and that recurrent or extensive thrombus was responsible for as many deaths as GI bleeding. It was concluded that oral anticoagulation therapy had a favourable benefit risk ratio in patients with an underlying pro-thrombotic disorder and it has been proposed that in such patients, or where there is a history of recurrent thrombosis, long term anticoagulation should be considered.

We report four patients with PVT, demonstrating an underlying coagulation defect, and or a recurrent thrombotic history, maintained on long term low molecular weight heparin (LMWH). These patients have been followed for up to 9 years with 3 monthly endoscopic banding, 6 monthly anti-Xa levels and yearly bone scan to detect osteopenia. Overall, there have been no episodes of significant GI haemorrhage, one patient developed a further thrombotic event, anti-Xa levels have been stable and one patient has asymptomatic osteopenia of the lumbar spine.

Long term management with LMWH may offer a less labile form of haemostatic control, in relation to oral anticoagulation. Whilst being effective and consistent, LMWH is also less problematic in patients with liver dysfunction.

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# **Quality of anticoagulation management in primary care practice**

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Currently an estimated 500 000 people in the United Kingdom receive oral anticoagulant therapy (OAT). The therapeutic window for OAT is narrow, with regular monitoring and dose adjustment required for effective safe treatment. Anticoagulants are one of the classes of drugs most commonly associated with fatal prescribing errors, whilst there is limited evidence of the quality of anticoagulant care delivered in primary care practice. The need for more stringent safety controls has been identified by the National Patient Safety Agency, with subsequent guidance in conjunction with the British Committee for Standards in Haematology (BCSH 2006).

Within Cornwall OAT is mainly managed in the primary care setting. To assess the delivery of anticoagulation services, a

questionnaire was developed with reference to the BCSH guidance and sent to the anticoagulant lead in 76 general practices in Cornwall. Thirty-two (42%) of surgeries responded, most commonly supervising anticoagulant care for 80–100 patients (range  $n = 20–220$ ), with results as tabulated below.

Among the respondents there was a high level of compliance with the BCSH guidance, with comprehensive governance programmes, indicative of good quality care. Ideally, all commissioned services should be assessed for compliance with current guidance, whilst systematic collection of outcome data and analysis of cost effectiveness would be informative.

	Percentage of surgeries
Computerised prescribing	94
Alert for co-prescribed drugs	83
Patient education	100
verbal and leaflet instruction	72
Audit of adverse outcomes	87.5
Audit INR time in range	75
Review of practice at clinical governance meetings	88

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# **Optimising the use of the clotting screen**

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The coagulation profile is often used as a screening test to detect occult coagulopathies, especially in the pre-operative setting. It is well known that in the absence of a personal or familial bleeding history, this test is of low yield and is more likely to raise incidental laboratory abnormalities of little clinical significance.

In light of recent financial pressures on our laboratory and the escalating use of the coagulation profile (APTT and PT in our laboratory), the use of this test over a period of one week was audited against existing laboratory guidelines. During this period, 642 coagulation profiles were performed (excluding patients on anticoagulants); equating to over 32 000 per annum. The audit showed that 372 (58%) of requests were found to be outside the recognised criteria for performing the test. During this period, only one clinically significant bleeding disorder was diagnosed (in a patient with a significant bleeding history).

Based on these results and after consultation with senior medical staff, it was decided to allow requesting of the coagulation profile only if it met appropriate criteria. New guidelines for the appropriate use of the clotting screen were drawn up and circulated to all medical staff. The laboratory began vetting all requests and rejecting those samples not fulfilling the guidelines. The use of clotting screens was then re-audited after 3 months, again over a 1-week period. There has been a significant drop in requests (336 requests) with an improvement in pattern of requesting (97/336 or 29% not indicated). There have been no reported unexplained bleeding episodes or attributable clinical mishaps in this 3-month period.

Restricting the use of the coagulation profile has not just resulted in financial benefits but has also improved medical practice and decreased unnecessary investigations and undue anxiety to patients.

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# **Evaluation of the CS-2000i analyser using coagulation, amidolytic and immuno-turbidometric assays**

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The CS-2000i, a new coagulation analyser from Sysmex, is capable of performing coagulation, amidolytic and immuno-turbidometric assays. Transmitted light can be monitored at multiple wavelengths (340, 405, 575, 660, 800 nm), allowing more efficient clot detection in the presence of interfering substances. The CS-2000i also offers a pre-analytical check and flagging system for haemolysis, icterus and lipaemia (HIL check). We evaluated this new analyser against the CA-1500 (light scatter) and STA Compact (mechanical) devices, using 200 samples from normal subjects and patients with icteric (bilirubin <489 µmol/l), lipaemic (cholesterol <8 mmol/l, triglycerides <7.5 mmol/l), or haemolysed plasmas (plasma Hb 0.4–6.1 g/l), and high or low fibrinogen content (>5 or <0.5 g/l). The CS-2000i was able to detect excessive icterus and haemolysis at 405 and 575 nm respectively and potentially flag these sample problems. Biphasic clot waveforms could be detected in some samples. Between day assay imprecision ( $n = 10$  replicates on each of 5 days) was assessed using commercial QC material. Low imprecision was observed for clotting tests (PT, APTT, Clauss fibrinogen [Fg]) with normal ( $cv < 0.83\%$ ) and abnormal ( $cv < 2.39\%$ ) plasmas; chromogenic assays (PC, AT-III, Plg) with normal ( $cv < 1.49\%$ ) and abnormal ( $cv < 3.37\%$ ) plasmas; immuno-turbidometric assays (D-Dimer) with 560 µg/l ( $cv 2.32\%$ ) and 2500 µg/l ( $cv 2.24\%$ ) plasmas. PT (INR 1.0–5.0), APTT (27–115s) and Fg (0.5–12.5 g/l) showed good correlations with the CA-1500 ( $r = 1.00$ ; 0.99 and 0.99 respectively) and STA Compact ( $r = 1.00$ ; 0.97 and 0.98 respectively). PC (15–270 U/dl), AT-III (20–180 U/dl) and Plg (17–200 U/dl) showed good correlations with the CA-1500 ( $r = 1.00$ ; 0.99 and 0.98 respectively). D-Dimer (0–1500 µg/l) showed good correlations with the CA-1500 ( $r = 0.95$ ). A new clot detection algorithm avoided early reaction errors and the facility to switch analysis to different wavelengths avoided interference by lipaemia, icterus and haemolysis, while fibrinogen sensitivity was excellent. While chromogenic and immuno-turbidometric assays exhibited good correlation with the CA-1500 analyser.

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# **The UK National External Quality Assessment Scheme (UK NEQAS) for molecular genetic testing in haemophilia**

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The identification of the causative mutation in individuals with haemophilia and other inherited bleeding disorders is common practice. In the UK, participation in a recognised external quality assurance scheme is a requirement for laboratory accreditation. In 1998, UK NEQAS (Blood Coagulation) established the first pilot

scheme to provide external quality assurance for molecular genetic testing in haemophilia. Results from three initial surveys highlighted problems with the quality of DNA and whole blood samples when used as QA material.

In 2003 the scheme was re-launched and addressed three aspects of genetic testing: the identification of a mutation, the interpretation of the mutation in the context of the clinical data and the reporting of results.

Between 2003 and 2006, five exercises involving whole blood or DNA (from immortalised cell lines) were circulated to participating centres in the UK and Iran. Laboratories were asked to undertake genetic testing, construct a report and return this within 6 weeks of receipt of samples. Reports were anonymised and scored in three areas (clerical, genotyping and interpretation) using a standardised template. Individual laboratory performances together with a summary of the exercise were returned to participants.

In 2003, a paper exercise highlighted problems with laboratory reports but following feedback to participants, only a single clerical error has been made in the five subsequent exercises. No laboratory has failed to identify the presence of absence of a mutation although inconsistencies in the interpretation have been noted.

Participating laboratories receive QA material every six months. Immortalised cell line material was introduced in 2005 and has been shown to perform well. Feedback from participating laboratories has been positive and overall the scheme has led to a more uniform approach for the reporting of genetic data and a more widespread use of standardised mutation nomenclature.

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# **Myocardial infarction (MI) in a 28 year old woman following treatment with DDAVP and tranexamic acid**

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DDAVP and tranexamic acid have been safely used in large numbers of patients with mild bleeding disorders. Large randomised studies support use of tranexamic acid in cardiac surgery to prevent intra- and peri-operative myocardial ischaemia and infarction. We report MI following therapy in a young woman without significant coronary artery disease.

*Case:* A 28-year-old woman with a platelet function disorder was given a single dose of DDAVP (0.3 mcg/kg) to allow an arthroscopy to be performed. This was followed by seven days therapy with oral tranexamic acid (1g tds). The platelet function disorder had been shown to be responsive to DDAVP and she had received DDAVP following the birth of her child and also routinely used intranasal DDAVP for control of epistaxis. 10 days after the operation she presented with severe central chest pain. ECG and elevated troponin I (27.81 µg/l) confirmed an inferior myocardial infarction. Immediate angiography revealed occlusion of a branch of the right coronary artery, too small to stent. The other coronary vessels were normal. Treatment was with aspirin and clopidogrel, and the patient made an uneventful recovery.

MI has been reported previously following DDAVP therapy. This effect is usually immediate and in elderly patients with significant atheroma. Mannucci (1993) did not find a significant excess of MI in patients receiving DDAVP during surgery. The timing of MI in our case makes it unlikely that DDAVP was responsible. Previous reports of arterial thrombosis associated with tranexamic acid are extremely rare. Only two cases of MI attributed to its use have been reported,

one in a 77-year-old and another in a 42-year-old recently initiated on the combined oral contraceptive. Both women were found to have coronary stenosis at angiography in the affected territory. Although the development of the thrombosis in this case is unusually late in relation to therapy, it seems unlikely that the MI would have occurred otherwise. These agents should not be regarded as entirely safe even in patients with low cardiovascular risk.

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### **Introduction of a biomedical scientist led bleeding disorder clinic**

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Basingstoke and North Hampshire Foundation Hospital supports a Haemophilia Comprehensive Care Centre where patients suffering from abnormal bruising or bleeding are referred for diagnosis and clinical management of their condition. Traditionally, all patients were seen in a bleeding disorder clinic by a Consultant Haematologist.

With the pressures on Consultant Haematologist time and vacancies, innovative solutions were required to continue to provide a quality service. With the extensive knowledge of the biomedical scientists within the haemostasis laboratory it was decided to utilise this in a new area of clinical activity and introduce a Biomedical Scientist led Bleeding Disorder Clinic.

With this practice new out-patient referrals are seen by the Biomedical Scientist where history is taken and appropriate laboratory investigations performed. All patients are now screened for both coagulation and platelet function at the first visit and further investigations instigated by the biomedical scientist according to test results and history knowledge. Following this the patient is a) discharged or b) returns to see the biomedical scientist for further tests. On completion of investigations the Consultant Haematologist sees either patients with an identified abnormality to discuss diagnosis or with an impressive history with no specific abnormality identified.

Over 12 months 60% of patients have been discharged after initial investigations, with 40% requiring follow-up, of which 50% were identified with VWD and 20% with a platelet abnormality.

This new model of service has streamlined investigation of potential bleeding disorders. Patients require less clinic visits to complete their investigations. It has extended the role of the biomedical scientist and made effective use of the Consultant Haematologist clinic caseload for patients with a diagnosed abnormality or clinically complex cases.

This service development was recognised by the Department of Health at the Healthcare Science Award 2006, where Dr Needham received the award for Innovation in Workforce Modernisation

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### **Management and outcome of pregnancy in patients with thrombophilia and a poor obstetric history at a single tertiary referral obstetric unit**

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The management of women with a history of recurrent miscarriage or late pregnancy loss and thrombophilia remains controversial. We performed a retrospective study from 1997 to 2005 of women with a poor obstetric history and a laboratory thrombophilia treated with

low molecular weight heparin (LMWH). The data was collected using clinical notes and computerized records. Data was available for 159 women between the ages 16 and 44 years: 66 patients had antiphospholipid syndrome; 24 factor V Leiden; 36 protein C/S deficiency; four antithrombin deficiency; four PT20210A prothrombin gene mutation and 25 were classified as miscellaneous with diagnosis including MTHFR variant, borderline APC resistance, elevated factor VIII, and factor XII deficiency. Overall 73 women were managed with LMWH only and 85 had both aspirin and LMWH (1 not documented). Of the 159 women, 118 had not had a previous successful pregnancy and of these 88 (75%) were then successful, 23 unsuccessful and seven not documented. Of 41 women with at least one previous successful pregnancy, 35 (85%) went on to have a successful outcome with treatment. The overall success rate for women with antiphospholipid syndrome and other thrombophilic disorders was 71% and 82% respectively.

We also studied 103 women with the same obstetric history as the patient group but with no documented thrombophilic defect and otherwise normal investigations including cytogenetics, who did not receive any LMWH. Of 61 women with no previous successful pregnancies 43 (70%) went on to have a successful outcome and in 42 women with a previous successful pregnancy, 35 (83%) went on to have a successful outcome. Most women at our centre with a poor obstetric history have a successful outcome in subsequent pregnancies whatever the laboratory defect or treatment given. Results from prospective randomized controlled studies are required to inform future practice and avoid unnecessary interventions.

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### **Management of life threatening bleeding in patients on warfarin – the use of prothrombin complex concentrate in a district general hospital setting**

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Warfarin is the most commonly used oral anticoagulant in the UK. The main side effect of warfarin is haemorrhage. In major/life threatening bleeding rapid reversal is necessary. The British Committee for Standards in Haematology (BCSH) guidelines recommend reversal of anticoagulation in patients with major bleeding with a factor concentrate in preference to fresh frozen plasma, together with 5 mg intravenous vitamin K.

We undertook a retrospective analysis over a 4 year period of the use of prothrombin complex concentrate (PCC) in the treatment of patients with life threatening bleeding. A total of 52 patients received PCC, 29 men and 23 women. Seventeen out of fifty-two patients were less than 70 years of age. The commonest indication for warfarin was atrial fibrillation (29), followed by prosthetic heart valves (8) and venous thromboembolism (7). A total of 19/52 patients had an INR within the appropriate target range. Eight patients had an INR of greater than 10. The commonest indication for the use of PCC was intracranial bleeding (19). The remaining 33 patients had life threatening bleeding at other sites, or had significant surgical bleeding. Twelve patients were treated with PCC alone, 33 patients received vitamin K and PCC. In seven patients it was not possible to ascertain whether they had received vitamin K. Only 8/52 patients were treated according to the recent updated BCSH guidelines, the remainder receiving additional FFP or vitamin K orally and/or in a different dose. In terms of immediate outcome, 18/52 patients died. In the longer term, looking specifically at disability, 26/52 patients made a good recovery, 4/52 patients had a moderate disability. Overall, 13/17 patients <70 years and 20/35 >70 years were alive.

We intend to use this data to produce local guidelines for the use of PCC and reversal of oral anticoagulants, and then re-audit management of warfarin reversal.

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# **Which patients with venous thrombosis do not require screening for malignancy? A predictive model to identify patients with VTE at minimal risk of malignancy**

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Association between VTE and cancer has been recognised for over a century. The incidence of occult or overt malignancy in patients with thrombosis is 7–26%. We propose here a predictive model using age, quantitative D-dimer level along with site of thrombosis. This study included 696 (M: 358; F: 338) patients from the prospectively maintained database of patients with venous thrombosis at a Teaching Hospital, between 2001 and 2005. All Patients with thrombosis received standard treatment with low molecular weight heparin and warfarin. A logistic multivariate regression model was fitted with an indicator variable for a subsequent cancer as the response and age, the natural logarithm of the quantitative D-dimer level and the site of the thrombosis as explanatory variables. The fitted model was validated using an additional set of independent data.

The model correctly identified the VTE patients without malignancy in with 98.5% accuracy. But the model was ineffective in identifying VTE patients with malignancy (9% accuracy). The area under the ROC curve was 0.72, indicating that the test developed for predicting cancer for the model data was reasonably good. Our model shows that below a predicted probability of 0.10 less than 5% of the patients actually developed cancer (9/190) whereas for 0.19 less than 10% of patients actually developed cancer (27/276).

In the validation dataset of 93 patients with VTE, the model correctly identified the number of patients without malignancy with 98.6% accuracy. There were no significant difference in the number VTE patients with cancer between the model and the validation dataset for the predicted probabilities of 0.10 and 0.19 (One-sample binomial tests; *p*-values of 0.650 and 0.246, respectively). Our model is useful for identifying patients at minimal risk of having malignancy with VTE. This model is reproducible as it has been validated by an independent dataset. This model will enable a focused and a cost-effective strategy of screening for malignancy in patients with VTE.

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# **Plasma thrombomodulin levels are significantly raised in pre-eclampsia**

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Thrombomodulin (TM) is an endothelial cell membrane glycoprotein which functions as a thrombin receptor. The thrombin-thrombomodulin complex initiates the protein C anticoagulant pathway. It activates protein C rapidly which together with protein S inactivate factor Va and factor VIIIa. Pre-eclampsia (P-EC) is a

complex multisystem disorder characterized by hypertension, proteinuria and edema. It occurs after the 20th week of pregnancy. P-EC has no cure, except by pregnancy interruption. In more severe cases, conditions such as eclampsia, HELLP syndrome or disseminated intravascular coagulation (DIC) may develop. The aim of this study was to assess plasma TM levels in pre-eclamptic women. Plasma TM levels were measured using an enzyme-linked immunosorbent assay (ELISA). A total of 57 subjects were studied. These include non-pregnant women (*n* = 22), healthy pregnant women (*n* = 15), and pre-eclamptic women (*n* = 20), at the third trimester. The mean and standard deviation (mean  $\pm$  SD) for the three groups were: non-pregnant women (0.609  $\pm$  0.311), healthy pregnant women (0.692  $\pm$  0.267) and pre-eclamptic women (0.917  $\pm$  0.324). Plasma TM levels showed a statistically significant difference when women with P-EC were compared to the non-pregnant women group (*P* < 0.05). However, we observed no significant difference when the pre-eclamptic women group was compared with the healthy pregnant women group. In conclusion, plasma TM levels are significantly elevated in women with P-EC. Endothelial cell injury and/or inflammatory reaction could have resulted in the increased plasma TM levels seen in our study. This finding may have a significant clinical ramification in the management of such patients. Detailed studies are required to address such an important relationship further.

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# **Venous thrombosis (VTE) has an adverse impact on the survival in patients with malignancy**

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Association between VTE and cancer has been recognised for over a century. Data on adverse impact of venous thrombosis on survival in patients with malignancy is conflicting. Study included 902 (M:463; F:439) patients from the database of patients from UK venous thromboembolism registry (VERITY). Counterpart group included 2263 (F: 1518; M: 745) consecutive patients without venous thrombosis from one site. D-dimer assays were done using Bio-Merieux kit. Median age at presentation was 66 years. Median D-dimer level was 2500  $\mu$ g FEU/ml. 17.3% had D-dimer > 8000  $\mu$ g. Sixty-one per cent had above knee and 34% had below knee VTE. Five hundred and twenty-two patients had no malignancy, 89 had bowel, 61 prostate, 56 breast, 41 gynaecological, 29 lung and 102 had miscellaneous carcinoma. Median follow-up was 21 months. Mean overall survival(OS) in non-VTE patients without malignancy was 56 months as compared to 54 months in VTE patients with malignancy. Mean OS in VTE patients with ca breast was 34 months (counterpart group: 47m). Median OS in VTE patients with ca bowel was 9 months (counterpart group: 36m). Median OS in VTE patients with ca prostate was 31 months (counterpart group: 33m). Median OS in VTE patients with gynaecological ca was 17 months (counterpart group: 50m). Median OS in VTE patients with miscellaneous carcinoma was 9 months (counterpart group: 30m). Median OS in VTE patients with ca lung was 5 months (counterpart group: 4m). Median D-dimer levels in VTE patients without malignancy, Ca Breast, Ca bowel, Ca Prostate, Gynaecological Ca, Miscellaneous Ca

and Ca Lung respectively were 2200, 3650, 4100, 2850, 3140, 3230 and 3400 µg FEU/ml. D-dimer > 8000 µg was associated with shorter survival (Log rank test;  $p$  value < 0.001). Our study shows occurrence of VTE shortens the survival in patients with malignancies. Our study also shows D-dimer > 8000 µg is associated with significant shorter survival. More studies are warranted to determine whether this adverse impact correlates with the thrombogenesis of underlying malignancy and also can it be negated by optimum anticoagulant therapy.

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### Platelet function testing: practice amongst UK NEQAS for Blood Coagulation participants, 2006

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Despite the importance of platelet function testing in investigation of bleeding tendency, there is little standardisation and no effective quality control of these tests. A questionnaire distributed in March 2006 sought to establish platelet function testing practice amongst UK NEQAS Blood Coagulation participants.

One hundred and fifty-eight centres reported use of one or more tests of platelet function. 27% reported the bleeding time as the only platelet function test they perform. A variety of bleeding time devices is employed. Reference ranges, primarily literature derived, ranged from < 6 to < 10.5 min. Thirteen per cent perform a combination of bleeding time (BT), PFA100 analyser and aggregometry tests, 20% BT + aggregometry, and 18% PFA100 + aggregometry tests.

Seventy-two centres employ the PFA100; 57/72 may not perform further investigations if a normal result is obtained. Sixty six out of seventy centres employ collagen/epinephrine cartridges with each patient, compared to 48/72 with the collagen/ADP cartridge. Marked variation in quoted reference ranges were observed, whether locally determined or literature derived. Fifty centres do not employ controls for this test.

For platelet aggregometry, variability in preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP) was accompanied by adjustment of the PRP platelet count, generally with PPP, in 73/79 centres. Maximum acceptable time for testing samples after collection ranged from 45 to 240 min. Forty nine out of eighty centres employed five different agonists in their investigations. Marked variability in instrumentation and agonist source was identified; eg 17 different sources of ristocetin were reported. Variability in agonist final concentrations was compounded by different units employed. 86/145 centres employed quantitative aggregation (48/86 reporting maximal aggregation); the remainder used visual estimation for qualitative assessment. Fewer than 10% performed other tests of platelet function. In conclusion, there is marked variability in the laboratory evaluation of platelet function; updated guidelines and standardisation of methodology are required.

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### Endocrine and CNS malignancies are strongly associated with symptomatic venous thromboembolism: findings from the VERITY registry

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The association between VTE and cancer is well recognised but VTE rates in specific cancers and the impact of VTE on mortality are poorly characterized. VERITY, an ongoing UK prospective VTE treatment registry, has enrolled more than 60 000 patients with suspected or confirmed VTE. To further elucidate the relationship between VTE and cancer, we compared VTE rates in cancer and non-cancer patients and compared the prevalence of different cancer types in VTE-positive and VTE-negative cases. This analysis was undertaken as part of a larger research program to characterize thrombosis risk and outcome in patients with cancer.

By Dec 2006, VTE diagnosis was known in 52 363 patients (27.1% [ $n = 14 199$ ] confirmed with VTE and 72.9% [ $n = 38 164$ ] with VTE excluded) and in 3437 patients with cancer. More patients with VTE had a diagnosis of cancer than VTE-negative patients – 13.7% (1939/14 199) vs 4% (1498/38 164), respectively. Comparing cancer to non-cancer patients, VTE was two-fold higher in cancer patients (56% [1939/3437] vs 25% [12 260/48 926], respectively).

Certain tumour types were more strongly associated with VTE. The prevalence of endocrine tumours was 16-fold higher in VTE patients than VTE-negative patients; CNS tumours were 14-fold more prevalent. Other malignancies were also associated with VTE – head and neck (10.3-fold higher in VTE patients); pancreatic (9.7); upper GI (7); lung (5.2); myeloma (4.6); bone (4.4); colorectal (4); leukaemia (3.5); gynaecological (3). Although breast and prostate cancer accounted for the highest number of cancer patients with VTE, these malignancies were only 2.8- and 2.6-fold more prevalent in VTE patients than VTE-negative patients, respectively. Melanoma, urological cancers and lymphoma showed a 2-fold higher prevalence in VTE patients.

These results confirm earlier findings that the overall risk of VTE in cancer patients is about twice the risk of patients without VTE and that endocrine and CNS malignancies are strongly associated with symptomatic VTE.

## Poster Presentations: Lymphoid Malignancy

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### B-cells from lymphocytic leukemias are capable of synthesis of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF)

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Angiogenesis is a crucial event in the survival and progression of solid as well as hemopoietic malignancies. To determine whether

angiogenesis in lymphocytic leukemias is an intrinsic property of leukemic cells, Bone marrow mononuclear B-cell lineage were separated from 25 acute lymphoblastic leukemia (ALL) and 15 Chronic lymphocytic leukemia (CLL) and cultured for 24 hour. Culture supernatant were assayed for the secretion of VEGF and b-FGF using enzyme linked immunosorbent assay. These data demonstrated that leukemic B-cells isolated from CLL and ALL patients at diagnosis spontaneously secrete both VEGF and b-FGF (55.17.0–295, 68.25.0–400.0, respectively) in CLL and (70.4; 20.4–130; 99.137.4–172.0, respectively) in ALL. The pretreatment bFGF and



VEGF levels were significantly higher as compared to that in remission both in ALL and CLL. The level of the secretion at diagnosis was higher in CLL-cells separated from advanced RIA stages as compared to those with favorable RIA stage ( $P < 0.05$  for both). On the other hand their levels did not differ in ALL FAB subtypes ( $P > 0.05$  for both).

In summary, these data collectively indicate that angiogenesis in lymphocytic leukemias likely to represent an intrinsic property and that the level of angiogenic factor secretion is correlated with advanced CLL disease.

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# **Incidence and significance of the $t(14;19)(q32;p13)$ in B-cell lymphoproliferative disorders**

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The  $t(14;19)(q32;p13)$  which deregulates BCL3 as a consequence of its juxtaposition to the IGH locus is a rare abnormality in B-cell lymphoproliferative disorders (LPD). It is said to occur primarily in CLL where it is associated with an atypical immunophenotype, typically the lack of CD23 expression and possibly a poor outcome. It has however been described in a number of other lymphoma subtypes. In order to clarify this we have evaluated a large series of B-cell lymphomas with interphase FISH. Samples were initially screened for IGH rearrangements using a dual colour IGH break-apart probe set (Vysis, 32-191 019) and cases with a split signal were further evaluated with a BCL3 dual colour breakapart probe set (Dako, Y5411). Cases of follicular lymphoma and mantle cell lymphoma were specifically excluded as they contain disease defining IGH translocations. Ninety-seven cases of diffuse large B cell lymphoma were evaluated and a  $t(14;19)$  was demonstrable in two patients both of whom had a preceding history of CLL. Interestingly in one of these patients the translocation was not demonstrable in the CLL cells prior to histological transformation. Forty-seven patients with extranodal marginal zone lymphoma (ENMZL) were also evaluated and a  $t(14;19)$  was demonstrated in a single patient with gastric MZL (overall incidence 2.1%). The translocation was not however demonstrable in CD5- LPD ( $n = 136$ ) but was seen in 2/76 (2.6%) patients with CD5+ CD23- LPD. The majority of these patients were considered to have atypical CLL on the basis of their clinical features, immunophenotype and lack of a  $t(11;14)$ .

We would therefore conclude that the  $t(14;19)$  is indeed a rare abnormality. It is seen in a minority of patients with atypical (CD5+ CD23-) CLL and may be associated with large cell transformation. It is also demonstrable in a minority of patients with ENMZL.

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# **Rearrangements of MALT1, IGH, FOXP1, BCL3 and PAX5 are rare events in extranodal marginal zone lymphoma in the UK**

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A number of balanced translocations have been described in extranodal marginal zone lymphoma (ENMZL). The  $t(11;18)$  and variant  $t(14;18)$  which deregulate MALT1 are the commonest reported occurring in up to 40% of patients. The  $t(3;14)$ ,  $t(1;14)$  and  $t(14;19)$  which deregulate FOXP1, BCL10 and BCL3 respectively have been

described in a variable proportion of patients. The purpose of this study was to evaluate the incidence of such abnormalities in the routine diagnostic setting in a geographically defined region of the UK. We therefore reviewed a series of 64 cases presenting between 11/03 and 12/06 in the Yorkshire and Humberside Haematology Networks. All cases were investigated for MALT1 rearrangements with an alpha satellite 18 control (Vysis 32-190055/ D18Z1) and cases with available biopsy material were further investigated for rearrangements of IGH (Vysis 32-191019), PAX5 (Dako Y5413), BCL3 (Dako Y5411) and FOXP1 (in-house probe). Seven out of sixty four (11%) cases demonstrated rearrangement of MALT1, 5/7 were of gastric origin. 16/64 (28%) cases demonstrated trisomy 18. Ten of fifty-six (18%) cases showed rearrangement of IgH which was attributed to a  $t(3;14)(p14;q32)$  in one case and a  $t(14;19)(q32;p13)$  in another. No cases (0/45) were demonstrated to have rearrangement of PAX5, but 11/45 (24%) showed three copies of PAX5 and similarly 16/42 (38%) had extra copies of FOXP1, consistent with complete or partial trisomy of chromosomes nine and 3 respectively.

The incidence of MALT1 rearrangements in this study is lower than most published series. We similarly failed to demonstrate rearrangements of PAX5. The  $t(14;19)$  and  $t(3;14)$  were both demonstrated but at low frequency (2.1% and 2.4% overall). It is clear that although significant advances have been made in our understanding of the pathobiology of ENMZL the underlying cytogenetic defect remains unknown in the majority of patients.

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# **Bone marrow fibrosis is a negative predictor for osteolytic lesions in patients with myeloma**

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Myeloma is associated with an unbalanced bone remodelling leading to osteolytic lesions. Marrow interstitial fibrosis is common in myeloma. Correlation of marrow fibrosis with clinical parameters and survival is conflicting. We have analysed the impact of marrow fibrosis in 83 (F: 45; M: 38) patients with median age 67 years on osteolytic lesions and survival. Reticulin silver impregnation is employed as standard matrix stain. H & E slides were reviewed for degree and pattern of plasma cell infiltration. Marrow reticulin was quantified from Grade 0-4. Forty-two per cent had increased marrow reticulin (> grade 2). Haemoglobin <10 gms/dl was in 47% patients. Thirty-three per cent had hypercalcaemia. Fifty-one per cent had >2 osteolytic lesions at diagnosis. Fifty-five per cent had  $\alpha_2$ -microglobulin >4 mg/l and 22% had renal failure at diagnosis. Forty-seven per cent needed >1 line of therapy. Marrow reticulin was a negative predictor for osteolytic lesions ( $p = 0.001$ ). Marrow reticulin did not correlate with other laboratory parameters. Marrow reticulin, sex, BJP and number of osteolytic lesions at diagnosis did not impact on OS (Log Rank test  $p$  values: 0.6, 0.07, 0.6 and 0.4). Known poor prognostic markers Hb <10 gm/dl, hypercalcaemia, renal failure and elevated  $\alpha_2$ -microglobulin were associated with poor OS ( $p$  values: 0.02, 0.04, 0.004 and 0.002) with median follow-up 27 months. Number of osteolytic lesions and anaemia at diagnosis had impact on progression free survival ( $p$  values: 0.01 and 0.02) where as raised  $\alpha_2$ -microglobulin, marrow reticulin, hypercalcaemia and renal failure ( $p$  values: 0.07, 0.7, 0.9 and 0.35) had no impact. Marrow reticulin had no effect on requirement for >1 line of therapy ( $p$  value: 0.17). Marrow reticulin is a negative predictor for osteolytic lesions, even though it does not appear to impact on survival in patients with

myeloma. Our study also confirms the poor prognostic role of anaemia, hypercalcaemia, renal failure and raised  $\alpha_2$ -microglobulin in patients with myeloma. Further study of the hgf/c-Met interaction may identify the negative correlation between marrow fibrosis and osteolytic lesions. This may enhance understanding of skeletal prophylaxis in patients with myeloma.

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### Ultra low dose thalidomide in myeloma

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Thalidomide is effective alone or in combination with chemotherapy for myeloma. The optimal dose is unclear. Thalidomide has a significant side-effect profile that may be dose related. Where patients could not tolerate thalidomide 50 mg daily, we tried an ultra-low dose (ULT) of 25 mg daily, alone or in combination with dexamethasone and/or cyclophosphamide.

Records were analysed of all patients who had received ULT for at least 2 months. Six patients were identified, median age 74.

Three of the six patients showed a marked reduction in biochemical markers of myeloma on ULT (decrease in paraprotein of 53%, 47%, serum-free light chains (FLC) of 98%). One of these patients was on ULT alone, while the other two were also on cyclophosphamide + dexamethasone.

One patient had already achieved a 90% reduction in FLC on 50 mg thalidomide + cyclophosphamide + dexamethasone over 6 weeks, and this response was maintained on ULT + cyclophosphamide + dexamethasone.

Two patients showed marginal biochemical responses to ULT + cyclophosphamide or + cyclophosphamide + dexamethasone (fall in paraprotein 26%, fall in FLC 27%) but neither had improved responses to Thalidomide 50 mg with cyclophosphamide  $\pm$  dexamethasone.

WHO performance status (PS) improved in our cohort from a median PS of 2.5 at onset of treatment to 1.5 at assessment date. No patients reported progression of neuropathy or unmanageable side effects when receiving ULT.

In summary, one patient had a 50% fall in paraprotein on ULT alone, suggesting that ULT may have activity in myeloma. The other 5 patients all had dexamethasone and/or cyclophosphamide with ULT, so it is not possible to claim that the anti-myeloma activity was due to ULT. However, two of these five patients had a good response and one continued in plateau, suggesting that the regime of ULT with dexamethasone and/or cyclophosphamide is active in elderly patients. This is important as some patients are unable to tolerate higher doses of thalidomide.

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### Genome complexity in patients with B-lineage acute lymphoblastic leukaemia revealed by array-based comparative genomic hybridization

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Array-based comparative genomic hybridization (aCGH) allows the entire genome to be scanned for DNA copy number alterations (CNA) at high resolution and mapped onto the human genome

sequence. Here, we report the analysis of CNA in ALL patients ( $n = 140$ ). We have targeted three distinct patient groups: those with no established chromosomal abnormality (group A,  $n = 80$ ); those positive for the ETV6-RUNX1 fusion (group B,  $n = 40$ ) intrachromosomal amplification of 21q (iAMP21, group C,  $n = 30$ ). To confirm and further refine abnormalities detected by aCGH, molecular copy number counting (MCC), and breakpoint cloning were carried out. CNA were common in all sub-groups, with group A patients showing recurrent involvement of chromosomes 6, 9, 12, 15 and 21. Deletions of 6q were heterogeneous, ranging from 34.5 to 91.1 Mb. They shared a common deleted region (CRD) of 1.8 Mb, including the GRIK2 gene. Deletions involving 9p ranged from 0.7 to 36.7 Mb in size and showed considerable heterogeneity in both the extent and location of the deletion. Deletions of 12p, ranging from 0.7 to 20.9 Mb, all with a CRD between 10.9 and 11.6 Mb, encompassing the ETV6 gene. Group 2 patients showed deletions of 11q and gain of Xq, in addition to recurrent imbalances of 12p and 21q. In Group 3, it was possible to accurately map chromosome 21 CNA showing considerable heterogeneity. This is the first report of genome wide detection of CNA in ALL patients using aCGH. It has provided further insight into the size, genomic position, gene content and high-resolution characterization of the breakpoints involved in these abnormalities, demonstrating that these changes are often more complex than anticipated. Such precise descriptions of the genetic alterations in ALL may lead to improved molecular diagnosis and prediction of outcome.

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### CD38<sup>+</sup> sub-clones exhibit increased proliferative activity but similar proliferative histories when compared with CD38<sup>-</sup> sub-clones derived from the same CLL patient

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CD38 expression is a marker of poor prognosis in B-cell chronic lymphocytic leukemia (CLL) but the biological rationale for this remains obscure. We recently showed that CD38<sup>+</sup> and CD38<sup>-</sup> sub-clones derived from the same patient have distinct gene expression profiles despite their monoclonality. Furthermore, we also demonstrated that CD38<sup>+</sup> sub-clones have an increased proliferative activity when compared with their CD38<sup>-</sup> counter-parts, as evidenced by elevated Ki-67 expression ( $n = 19$ ;  $P < 0.0001$ ). This finding raised the possibility that the CD38<sup>+</sup> fraction of individual patients may have different proliferative histories. Therefore, we measured telomere lengths and telomerase expression (hTERT) in cell-sorted CLL cells. hTERT expression was significantly higher in CD38<sup>+</sup> sub-clones when compared to CD38<sup>-</sup> sub-clones derived from the same patient ( $P = 0.03$ ) and the increased expression of hTERT was confirmed at the protein level by flow cytometry. However, the telomeres of CD38<sup>+</sup> and CD38<sup>-</sup> sub-clones were not significantly different from one another implying that they had undergone a similar number of cell divisions. Importantly, the telomere lengths of both sub-clonal populations were consistently short (approximately 3 Kb) indicating very active proliferative histories. Taken together, our data suggest that CD38 is not stably expressed on individual CLL cells but is more likely expressed in a dynamic fashion with higher expression found on those cells most recently exposed to activation stimuli. The fact that both CD38<sup>+</sup> and CD38<sup>-</sup> sub-clones have similarly short telomeres implies that CD38<sup>-</sup>

sub-clones have probably repeatedly expressed CD38 in their past (and may do so again). If this were not the case then CD38<sup>-</sup> sub-clones would have a permanently reduced capacity for proliferation their telomeres would be longer than those of CD38<sup>+</sup> sub-clones. Therefore, the poor prognosis associated with CD38 expression in CLL is probably caused by an enhanced proliferative capacity and the subsequent elevated risk of clonal evolution.

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# **Tissue samples involved by multicentric Castleman's disease among HIV-positive individuals is often involved by microscopic foci of Kaposi's sarcoma**

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Multicentric Castleman's Disease (MCD), a lymphoproliferative disorder and Kaposi's sarcoma (KS), a vascular tumour, both occur at a higher frequency among patients with human immunodeficiency virus (HIV) infection. Furthermore, the virus human herpes virus 8 (HHV8), with an ability to persist in a latent form in both B-lymphoid cells and endothelial cells, is causally associated with both MCD and KS. The co-existence of these two HHV-8-associated malignancies in same tissue samples has hitherto not been systematically investigated.

In this report, we compile the histological and immunohistochemical findings in seventeen consecutive cases of MCD seen during 2004 to Jan 2007, which included 15 lymph node (LN) and two spleen samples. All but two patients were men. The age range was 34–70 years. Values of plasma HHV-8 viral load was available in 10 cases; it ranged from 0 to 400 000 viral copies per mm<sup>3</sup> (median: 550). In addition to MCD, 11 of 17 (65%) samples showed evidence of co-existing KS. The foci of KS were typically 'microscopic' involving the LN capsule, trabeculae or the hilum. In all cases, presence of HHV-8 was documented in the so-called 'plasmablasts' by immunostaining with HHV-8 latent nuclear antigen-1 (LANA1). In cases involved by KS, the spindle cells showed expression of HHV-8-LANA-1 and of the endothelial markers CD34/CD31. Additional clinical information was available in 12 cases. Six of them had KS elsewhere; four of these six had microscopic evidence of KS in lymph nodes.

Co-existence of MCD and KS in the same tissue sample is a common phenomenon that has not been previously appreciated.

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# **Marginal zone B-cell lymphoma with prominent follicular colonisation - difficulties in diagnosis; a study of 15 cases**

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While colonisation of reactive follicles is well described in MALT lymphoma, this is not fully appreciated among nodal marginal zone B-cell lymphomas (NMZL). In this case series, histological features and immunohistochemical findings of fifteen NMZLs with prominent follicular colonisation is described.

Fourteen of the cases were referrals from other hospitals. All 15 cases had showed a follicular pattern, which was prominent in six

cases. Cytologically, the cells were small-cleaved cells with moderate to abundant cytoplasm. There was a prominent plasmacytoid differentiation in six cases.

A feature common to all cases was prominent 'follicular colonisation'. The process of colonisation varied in its extent between cases and between follicles. In many follicles the colonisation was partial and follicles were composed of a reactive germinal centre component as well. Follicular pattern was highlighted with CD21 and CD23 stains. Follicular colonisation was accentuated with the help of immunostains – CD20, CD10, Bcl-2, Bcl-6 and MUM1. The benign/reactive follicle centre cells expressed CD20, CD10 and Bcl-6 and were negative for Bcl-2 and MUM1. On the other hand, the colonising marginal zone lymphoma cells expressed CD20, Bcl-2 and often MUM1, and they were negative for Bcl-6 and CD10. Follicles, which were partially colonised, showed a typical 'moth-eaten' appearance on CD10, Bcl-2, Bcl-6 and MUM1 immunostains.

In none, excepting one of the referred cases a diagnosis of NMZL was made at the time of initial diagnosis. Initial diagnoses included follicular lymphoma, chronic lymphocytic leukaemia with proliferation centres, progressively transforming germinal centres, suspect low-grade lymphoma and reactive lymph node.

Recognising and appreciating that a subset of NMZLs present with a prominent follicular pattern where these structures represent follicles colonised by marginal zone lymphoma cells is necessary in reaching the correct diagnosis. Appropriate use immunohistochemistry and knowledge of immunohistology features can aid in the correct diagnosis.

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# **FDG-PET is highly sensitive for Burkitt's lymphoma and may predict relapse risk**

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Interim fluorodeoxyglucose (FDG) positron emission tomography (PET) scanning is highly predictive of relapse risk in both Hodgkins and non-Hodgkins lymphoma. We report the results of PET scans prior to and during intensive chemotherapy in patients treated for Burkitt's lymphoma at our centre.

Ten patients were identified. Median age was 34 years (range 15–49). Six patients (60%) were male. Two patients were HIV positive; two were on immunosuppressive medications post-renal transplant.

All patients had histological evidence of typical Burkitt's lymphoma. All patients were treated with intensive chemotherapy according to the French Society of Pediatric Oncology LMB89 protocol apart from one patient with completely resected stage 1 disease who received three cycles of CODOX-M chemotherapy.

The patient with complete surgical resection of disease had a negative pre-chemotherapy PET scan. All remaining patients had positive PET scans. Of these nine patients, the frequency of PET uptake at specific sites was: bone/marrow 78%, nodal 67%, liver 44%, gut 44%, spleen 22%, retroperitoneum 22%, renal 11%, breasts 11%, adrenals 11%. Presentation Ann Arbor stage was: stage IV in 80%, stage II in 10%, stage I in 10%.

Seven patients had interim PET scans within the first three months of chemotherapy. Six out of seven patients (86%) had a complete response on interim PET scan; one patient had minimal residual uptake. At a median follow-up of 39 months (range 8–144),

all six patients who had achieved complete response on interim PET are in complete remission. The patient who had minimal residual disease on interim PET subsequently progressed and died.

FDG-PET is highly sensitive for Burkitt's lymphoma and is of particular value in confirming early stage disease. Evidence from this small series suggests that early interim PET can differentiate between patients who are destined to be cured and those who will fail therapy.

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### Clinical significance of plasma endostatin in patients with chronic lymphocytic leukaemia

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Several studies have pointed to angiogenesis as a new potential prognostic factor in chronic lymphocytic leukaemia (CLL). Many papers have investigated the role of angiogenic activators in CLL. However, there is insufficient data regarding inhibitors of angiogenesis in this disease. Endostatin is a naturally-occurring 20-kDa C-terminal fragment of collagen XVIII and has a potent antiangiogenic and antiproliferative effects *in vitro* and *in vivo*. To assess potential prognostic role of this cytokine in CLL, we quantified plasma concentrations of endostatin using sandwich ELISA (RD Systems) in peripheral blood plasma of 44 patients with never-treated CLL and 26 healthy blood donors. Endostatin concentrations were significantly increased in CLL patients when compared to controls (mean  $\pm$  SD [standard deviation], 226.9  $\pm$  69.0 vs 171.0  $\pm$  9.1 pg/ml, 95% confidence interval of mean [CI], 205.9–247.8 vs 152.2–189.8,  $P = 0.0003$ ). There was no difference in endostatin levels between patients with stable ( $n = 18$ ) and progressive ( $n = 25$ ) disease. Similarly, no difference was found between patients with early vs intermediate vs advanced disease (Rai modified staging). In ten patients with serial endostatin measurements before and after intensive therapy using fludarabine-based treatment, there was a trend towards endostatin increase after therapy; however, the difference was not significant ( $P = 0.13$ ). We conclude that plasma endostatin might be useful as a part of complex assessment of angiogenesis in CLL. Further studies with regard to clinical significance and correlation to other angiogenic markers and modern prognostic factors (IgVH mutation status, genetic abnormalities) are necessary. Supported by grant NR/8373-3 from Ministry of Health of Czech Republic.

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### Relationship of ZAP-70 expression and circulating angiogenic cytokines in chronic lymphocytic leukaemia

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Chronic lymphocytic leukaemia (CLL) is a disease with an extremely variable clinical course. Several studies have shown that angiogenesis is increased in CLL and may potentially serve as a new prognostic factor. Zeta-associated protein of 70 kDa (ZAP-70) is an intracellular tyrosin kinase belonging to modern powerful prognostic markers in CLL with significant impact on clinical course. In our study, we

analyzed ZAP-70 expression using flow cytometry in CLL cells from peripheral blood of 32 patients. Furthermore, we quantified plasma concentrations of angiogenic activators (vascular endothelial growth factor – VEGF, basic fibroblast growth factor – bFGF) in peripheral blood plasma of the same CLL patient group and 80 healthy donors. Commercially available sandwich ELISA kits (RD Systems, MN, USA) were used for bFGF and VEGF measurement. ZAP-70 expression was quantified using PE-conjugated ZAP-70 monoclonal antibody (Caltag); cut-off level of 20% positivity was used as recommended by literature. Both angiogenic cytokines were significantly increased in CLL patients when compared to controls (bFGF,  $P < 0.0001$ ; VEGF,  $P = 0.0004$ ). Twenty patients were ZAP-70 negative and eleven ZAP-positive. Interestingly, bFGF and VEGF correlated inversely with percentage of ZAP-positive cells (bFGF,  $r = -0.43$ ,  $P = 0.014$ ; VEGF,  $r = -0.39$ ,  $P = 0.025$ ) and were significantly elevated in ZAP-negative patients (bFGF,  $P = 0.021$ ; VEGF,  $P = 0.035$ ). In conclusion, our findings underline the importance of angiogenic signaling in CLL and point to possible association with ZAP-70 expression. Further investigation in terms of impact on clinical course and survival is clearly warranted. Supported by grant NR/8373-3 and research project MZO 00179 906 from Ministry of Health of Czech Republic.

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### Use of bone marrow flow cytometry in multiple myeloma – an audit

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Bone marrow plasma cell assessment is a key component of myeloma diagnosis and treatment monitoring. Light microscopy, fluorescence-activated cell sorter (FACS) analysis and trephine histology are the established methods used to assess marrow plasmacytosis. Generally, sampling variation and techniques give differing estimations of marrow plasma cell involvement. Published studies indicate that FACS underestimates the degree of marrow plasmacytosis. This study has assessed the role of FACS in myeloma diagnosis and follow-up.

**Aims:** (1) Investigate the number of requests for FACS in marrow aspirate samples in diagnostic, follow-up and day 100 post-autograft cases (2) Determine if FACS results affect management decisions. (3) Compare the aspirate morphology results with FACS results.

**Methods:** A Cohort of patients attending the Southampton myeloma clinic over a 3-month period, who had marrow aspirates during the twelve months prior to 30 Sep 2006, was included. CD19, CD45, CD38, CD56 antibodies were used in two combinations for plasma cell FACS analysis. Aspirate morphology reports, FACS reports and documentation in patient records were reviewed.

**Results:** Sixty aspirates from 53 patients were analysed (number of FACS requests in brackets) as follows: diagnostic 11 (11); non-transplant follow-up 39 (34); day 100 post-autograft samples 10 (8). Results from FACS confirmed neoplastic plasma cells but did not influence patient management decisions from any of the 60 samples. In general, the percentage of plasma cells counted by FACS was lower than that counted by light microscopy in the majority of samples. The total estimated local cost of FACS analysis using primary screen and myeloma antibodies was £4835.

**Recommendations:** FACS did not affect management in both autograft and non-transplant follow-up samples. FACS screen in

diagnostic samples assists exclusion of other pathology and confirms neoplastic phenotype of the plasma cells.

**Conclusion:** Routine use of bone marrow FACS in myeloma follow-up is not indicated.

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**VH3-48 and VH3-53 gene rearrangements represent unique subgroups in CLL and are associated with biased lambda light chain restriction, homogenous LCDR3 sequences and poor prognosis**

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**Background:** In recent years IgVH mutational status, VH gene usage, and the potential role of antigens in the leukomogenesis of chronic lymphocytic leukaemia (CLL), have been studied extensively. In particular, the identification of VH3-21 gene usage as a unique subset of CLL has lead to questioning of the prognostic limitations of IgVH mutational status, as VH3-21 usage is associated with poor prognosis, irrespective of the fact that two thirds of such patients have mutated IgVH genes. Furthermore, specific gene usage has been linked with highly homogeneous heavy and light complementarity determining regions (CDR3), indicating that these patients possess virtually identical BCR binding sites and thus suggesting a common antigenic progenitor.

**Aims:** The aims of this study were to verify these findings in a larger cohort of CLL patients.

**Methods:** Three hundred patients were recruited from Belfast City Hospital Haematology Outpatient Clinic and surrounding regional hospitals. Clinical staging (Rai and Binet), immunophenotyping, lymphocyte doubling time (LDT) and time to treatment (TTT) were available on all patients. IgVH and IgVL mutational status, gene usage and CDR3 sequences were determined using multiplex BIOMED-2 primers and protocol and sequence analysis. FISH analysis was performed on all patients.

**Results:** VH3-48 ( $n = 14$ ) and VH3-53 ( $n = 6$ ) gene rearrangements showed biased lambda light chain restriction (12 $\hat{e}$ : 2 $\hat{e}$ , 4 $\hat{e}$ : 2 $\hat{e}$ , respectively). Further analysis revealed overuse of VL3-21 (V $\hat{e}$ 2-14) gene (7/8) and highly homogenous LCDR3 sequence (QVWDSSSDHPWV) in VH3-48 patients. Both VH3-48 and VH3-53 categories showed a preponderance of females, short LDT (<1-21 mths) and an absence of any poor prognosis chromosomal aberrations.

**Conclusions:** This study has confirmed earlier findings that VH3-48 rearrangements and VH3-53, represent unique subgroups in CLL associated with poor prognosis, irrespective of mutational status. The recurrent use of specific VL genes and homogenous LCDR3 sequences in these patients suggests a common aetiological factor.

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**Associations between gender, age at presentation, IgVH mutational status, gene usage, clinical stage and cytogenetic aberrations in B-CLL**

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B-cell chronic lymphocytic leukaemia (B-CLL) is a heterogenous disorder with a highly variable clinical course and median survival of approximately 10 years. It affects mainly older individuals and shows a male preponderance. The aims of this study were to determine if age at presentation, or gender, showed an association with IgVH mutational status and/or gene usage, clinical stage or cytogenetic aberrations. Three hundred and three B-CLL patients were recruited from Belfast City Hospital Haematology Outpatient Clinic and surrounding regional hospitals. Clinical staging (Rai and Binet), immunophenotyping, lymphocyte doubling time and time to treatment were available on most patients. IgVH mutational status, gene usage and CDR3 sequences were determined using multiplex BIOMED-2 primers and protocol and sequence analysis. FISH analysis was performed on all patients. Eighteen patients were <50 years of age and 285 >50 years of age upon presentation. There were no significant differences in the male:female ratio, IgVH mutational status, or poor prognosis cytogenetic aberrations between the two age groups. However, there was a significant increase in the number of Binet stage B and C patients in the older age category. In addition, IgVH4-34 gene usage was more prevalent in the younger age group (23.5% compared to 11.0% in the older group). Overall, the total CLL cohort consisted of 61.4% males and Binet stage B and C disease, and unmutated IgVH gene status, were significantly more frequent in males than females. Interestingly, IgVH3-30 and IgVH4-34 gene rearrangements were significantly more common in females than males. Females were also more likely to have no detectable poor prognosis cytogenetic aberrations, whilst trisomy 12 was significantly more prevalent in the male sub-group. In conclusion, our findings confirm that male CLL patients have biomarkers associated with poorer prognosis, a factor accentuated with advancing age at diagnosis. The associated molecular basis for this phenomenon remains to be elucidated. Ongoing studies are being carried out to investigate the biased IgVH gene usage and proportion of somatic hypermutation in the female sub-group.

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**Bortezomib, low dose intravenous melphalan and dexamethasone for patients with relapsed multiple myeloma: results of a phase I/II clinical trial**

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Bortezomib is an effective treatment for patients with relapsed multiple myeloma (MM) with an overall response rate

(MR + PR + CR) of 46% and time to progression of 6.2 months (APEX study).

In this multi-centre, non-randomised Phase I/II clinical trial, patients with relapsed MM received bortezomib 1.3 mg/m<sup>2</sup> on Days 1, 4, 8 and 11 of a 28 day cycle, and intravenous melphalan on Day 2 for a maximum of 8 cycles. The maximum tolerated dose (MTD) of bortezomib was 7.5 mg/m<sup>2</sup> as defined in the Phase I component. Dexamethasone 20 mg on the day of and the day after each dose of bortezomib was permitted for progressive or stable disease.

Fifty-three patients were enrolled (median age 61 years) with a median of three lines of prior therapy of which 39 (74%) have had one previous autologous stem cell procedure, five (9%) have had two, 34 (64%) have had prior exposure to thalidomide and five (9%) to bortezomib. The overall response rate (CR + PR + MR) was 76% rising to 80% (CR 10%; nCR 2%; VGPR 8%; PR 39%; MR 20%) with the addition of dexamethasone in 14 cases. Rapid responses were seen (median time to response 1 month) and the median time to progression was 10 months (median overall survival not yet been reached). Of the patients that had disease progression eight (30%) had responses of longer duration than their previous therapy.

Toxicities were acceptable with 22 SAEs reported (15 hospitalisations due to infection). The most common grade 3–4 adverse events were: thrombocytopenia (53%), neutropenia (49%), infections (17%) and neuropathy (11%) and 13 patients were withdrawn from study due to toxicity. Of note 11 patients (28%) had pre-existing grade 1 neuropathy prior to starting therapy.

This combination of bortezomib, low dose intravenous melphalan and dexamethasone is highly effective (ORR 80% 12% nCR/CR) and tolerable in patients with relapsed multiple myeloma.

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### An audit of presenting features, treatment and outcome in nodular lymphocyte predominant hodgkin's disease. A 10 year experience in the West of Scotland

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Retrospective data was collected from an audit of forty-two patients with a diagnosis of nodular lymphocyte predominant Hodgkin's disease (nLPHD) over a 10-year period (1997–2006) in the west of Scotland. This region has a population of 2.5 million. Detailing patient characteristics, presentation, treatment and outcome of nLPHD in comparison to national data for patients with classical Hodgkins disease. It confirms that the majority of patients present with Stage I or II disease, no B symptoms and with a median duration of lymphadenopathy prior to presentation of 6 months (1 month–18 months). The patients have a median age of 39 years with minimal co-morbidity. Overall survival is excellent with only one death which was unrelated to disease at a median follow-up of 55 months (1 month–204 months). Most patients were treated by radiotherapy alone, followed by chemotherapy alone. Three patients had combined modality therapy. Two patients had excision biopsy followed by simple observation. Two of the 42 patients are currently undergoing treatment. Of the 40 treated, 38 (95%) reached complete remission (CR). The remaining two patients remain in partial remissions that have lasted for 18 months and 36 months. Four (10%) patients relapsed and two (5%) developed DLBC NHL. Of the four patients that relapsed, two have gone into second CR and the other two are currently undergoing treatment. The main complication, so far, of radiotherapy was endocrine related and of chemotherapy was infection.

**Conclusion:** Nodular lymphocyte predominant Hodgkins disease is a clinically separate entity to classical Hodgkins disease often presenting at early stage, without B symptoms and has a good prognosis. No disease related deaths were identified in our patient population. It may therefore require less intensive treatment than classic Hodgkin's disease in order to reduce the potential late complications of therapy.

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### Haematological malignancies and palliative care

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Haematological malignancies are a diverse group of diseases which vary in terms of aggressiveness, symptoms, illness course and outcomes yet little is known about provision of palliative care for this group of patients. We report a 12-month pilot project whose primary aim was to describe the frequency and characteristics of patients with haematological malignancies who are (and are not) referred to palliative care, and the determinants of these processes. The project utilised the resources of the Haematological Malignancy Research Network (<http://www.bloodcancers.info/>) a collaborative venture between NHS clinical and diagnostic services and the Epidemiology and Genetics Unit at the University of York.

Data were abstracted from hospital records of 108 patients who died during the study period: 27 diagnosed with leukaemia (23 AML); 48 lymphoma (30 DLBCL); 22 myeloma; and 11 with myelodysplastic syndromes. Ninety-three patients (86.1%) were over 60 years of age, with 33 aged 80 years or older. The co-existence of one or more other illnesses increased with age from 20.0% in the group aged <60 years, to 25.0% at 60–79 years, and 39.4% in patients aged 80 years or over. Thirty-three patients (30.6%) were referred to specialist palliative care services. There was little difference by age or diagnostic group in patients referred, although those aged <60 years were less likely to be referred than older age groups. 25.4% of patients (17/67) dying on a hospital ward received specialist palliative care compared with 85.7% (6/7) dying at home and, unsurprisingly, all patients dying in a hospice (8/8). Time between diagnosis and death influenced referral: 46.2% (24/52) of those dying 30 days or more after diagnosis were referred to specialist palliative care compared with only 19.1% (8/42) of those dying within 30 days. In 14 patients diagnosis was made after death.

A 3-year project investigating patient needs has now commenced.

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### T-lymphocyte subsets in treated and untreated patients with B-CLL. Effect of treatment on T-cell subsets and correlation with infections

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T-cell dysfunction occurs in B-cell chronic lymphocytic leukaemia (B-CLL) and may contribute to the protean manifestations of the disease. CD8+ lymphocytosis correlates with CLL disease

progression and low expression of CD4 and CD8 is seen when autoimmune disease coexists. B-CLL patients at diagnosis with small tumour loads do not show signs of immune deficiency and some, treated with fludarabine-containing regimens have decreased inhibitory function of T-cells. We therefore analysed T-lymphocyte subsets by FACS Calibur flow cytometer (Becton Dickinson) and correlated this with clinical data in 20 patients with either untreated but known ( $N = 11$ ) or treated B-CLL ( $N = 9$ ) to evaluate the impact of treatment on T-cell subsets. B-CLL patients were studied on a single occasion in the period from May to July 2006 and correlations were made with clinical data. Analysis of the percentage and absolute numbers of total lymphocytes, total T-cells and T-cell subsets (pan T-cells (CD3), helper cell (CD4), suppressor cell (CD8), CD4/CD8 ratio, natural killer cells (CD56), total B cells (CD19), and pre B-cell (CD10) were performed and correlated with clinical data. The relationship of each antigen with lymphocyte count, age, treatment status (treated or untreated), drug regime and infection rate was tabulated against increasing numbers of each T-cell subset.

Results-A variation in absolute CD4 lymphocyte counts in both treated and untreated B-CLL patients was seen with an increase in absolute CD4 count in untreated patients even with small amounts of disease (absolute lymphocyte count  $8-89 \times 10^9/l$ ). Patients treated with intensive chemotherapy protocols revealed significant T-cell depletion with a more profound reduction in CD4 count than CD8 resulting in a significantly reduced/reversed CD4/CD8 ratio compared to untreated. In our study chemotherapy was found to be the primary contributor to the T-cell depletion observed in patients with BCLL. This is primarily overall T-cell subset depletion, with CD4 depletion being generally more severe than CD8 depletion. A probable correlation between T-lymphocyte subsets, numbers and the type of infection and number of infections was noted.

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### Spontaneous remissions and relapses of chronic lymphocytic leukaemia

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Chronic lymphocytic leukaemia (CLL) is characterised by the presence of clonal malignant lymphocytes in peripheral blood, tissue and organ infiltration and relentless albeit variable rate of progression. Isolated reports have described rare spontaneous remission of CLL. The purpose of this contribution is to report further cases of documented spontaneous remission and relapse of genuine CLL verified by immunophenotyping.

Five patients three males and two females with an age range of 65–77 years (at diagnosis) were identified with history of spontaneous remission(s) with or without relapse(s) during routine haematological follow up of 3–12 years from diagnosis in a small district general hospital serving a population of just under 100 000 population. Spontaneous remission was defined as reduction of lymphocytosis to below  $4 \times 10^9/l$  and resolution of lymphadenopathy without the use of cytoreductive therapy. The maximum lymphocyte counts prior to spontaneous remission were 22.7, 6.2, 7.3, 5.6 and 30.7 and the lowest lymphocyte counts following spontaneous remission were 1.5, 1, 2, 3.3 and  $1.4 \times 10^9/l$  (respectively). At diagnosis four patients were at Binet Stage A whereas the fifth patient was at Binet Stage B due to widespread lymphadenopathy. Three patients had lymphadenopathy at some stage of their illness. However, the most interesting case was that of a patient who had no lymphadenopathy at diagnosis but he developed bilateral cervical and inguinal lymphadenopathy 1 year later. Lymphadenopathy disappeared within a few months when he achieved spontaneous remission of CLL. However, lymphadenopathy

reappeared with profuse sweating when his CLL relapsed after just over a year of complete spontaneous remission.

In conclusion, spontaneous remissions and relapses of CLL are not as rare as commonly thought. Further studies are needed to identify and understand the factors responsible, if these are to be exploited therapeutically in CLL and possibly in other malignancies.

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### Is JAK2 V617F negative ET due to clonal lymphoid proliferations? A report of three cases of JAK2 negative sustained thrombocytosis in lymphoproliferative disorders mimicking co-existing essential thrombocythaemia

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Essential thrombocythaemia (ET) is a chronic myeloproliferative disorder (CMPD) of the stem cell however some patients with ET show polyclonal haematopoiesis. Only 50% of patients with ET show the JAK2 V617F mutation supporting the observation that ET is pathologically heterogeneous. Distinguishing clonal and reactive megakaryocytic proliferation has problems. A diagnosis of ET requires the absence of a reactive cause, characteristic bone marrow (BM) morphological features, degree and duration of thrombocytosis, and now JAK2 V617F positivity. ET megakaryocytes as defined by Thiele (2002) are thought to be characteristic with presence of increased numbers of giant to large megakaryocytes in loose clusters with mature, multilobulated staghorn-like nuclei, in a normocellular or mildly hypocellular marrow. Pajor et al in 2004 reported varying lymphoid populations in up to 32% of trephine biopsies of 106 patients with CMPD including 28 patients with ET. In 5% (four of 81) including one case of CML and three cases of CMPD (non specified) PCR analysis revealed clonal B-cell populations. We describe three female patients with lymphoproliferative disorders, two with documented CD5 positive clonal B-cells in the marrow with sustained thrombocytosis. Bone marrow trephines showed megakaryocytes akin to ET but absent JAK2 V617F. Hence a firm diagnosis of ET could not be established but in case 2 and 3 sustained rises in platelets to  $>1000 \times 10^9/l$  necessitated Hydroxyurea therapy which controlled platelets but had no effect on lymphoid cells. In case 1 however treatment with chlorambucil lead to reduction of platelets to normal levels. As ET and other lymphoproliferative disorders like CLL are 'common', by chance they may co-exist. Also megakaryocytic proliferations akin to ET may occur in the background of an established lymphoproliferative disorder. In such cases the megakaryocytes may be driven by the lymphoid neoplasm through a paracrine mechanism but mimic ET. Diagnosis of ET should be made carefully in the presence of JAK 2 negativity. Some cases may have indolent low grade clonal lymphoid populations not detectable by conventional means.

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**Childhood ALL minimal residual disease analysis by proteomics****EC Burt, F Hussain, G Wright, M Corbo, U Johansson, S Medahuni and FE Cotter***Department of Haematology, Institute of Cell and Molecular Science, Bart's and The London, Queen Mary School of Medicine, London, UK*

Acute lymphoblastic leukaemia (ALL) is the commonest childhood malignancy, accounting for approximately one-third of all childhood cancers. Treatment for the disease has substantially improved with long-term survival in nearly 80% of cases. However, current therapy is associated with significant side effects and 20% of patients are not cured despite intensified treatment.

The molecular measurement of minimal residual disease (MRD) can assist in the early evaluation of treatment, potentially leading to improved clinical outcome. The most specific and sensitive method for monitoring MRD is the polymerase chain reaction-based analysis of rearranged immunoglobulin and antigen-receptor genes. This method permits stratification of patients to different risk groups according to MRD level. However, the method cannot be applied to all samples, and is labour-intensive and costly. Patients could benefit from improved or additional methods of MRD detection. Liquid proteomics may be a means of identifying novel 'biomarkers' associated with risk groups.

A proteomic study of childhood ALL samples using the Ciphergen ProteinChip system was initiated. The system is ideal for protein profiling, with disease and control samples processed on the same chip surfaces and analysed under the same conditions. Subsequent spectra comparison can identify patterns of protein expression, to be used as 'fingerprint' profiles for the diagnosis, follow up and biomarker discovery for patients.

Plasma samples taken from pre-B cell ALL patients and age-matched controls were analysed, having been divided into high- and low- risk groups according to MRD level. They were assessed prior to treatment (day 0) and after the first round of induction therapy (day 28). Markers capable of discriminating between high- and low-risk groups were identified, including a 6 kDa marker with differential levels of expression between risk groups at day 0 and 28. These findings may provide an alternative MRD approach for ALL.

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**Dynamic assessment of apoptosis for *in vitro* design of bortezomib combination therapies for lymphoid malignancies****LC Wallis\*, MJ Streetly\*, R Auer\*, J Gribben\*, D Zhang†, WD Hankins‡, S Mehdi Zadeh† and FE Cotter\****\*Centre for Haematology, Institute for Cell and Molecular Science, Bart's and The London, Queen Mary's School of Medicine and Dentistry, London, UK, †Bio-Kinetic Profiling Ltd, London, UK, ‡Johns Hopkins University, Baltimore, MD, USA*

Conventional techniques for assessing drug response and apoptosis induction rely on static assessment of cellular changes at predetermined time points (e.g. detection of exposed membrane phospholipids by Annexin V). The Kinetics of Optical Response assay (KOR) is a new technique that detects induction of apoptosis dynamically. It detects changes in optical density associated with membrane blebbing related to growth and death, allowing detection of apoptosis in real time. This study uses the KOR assay in lymphoid malignancy and shows sensitivity to apoptosis induction by conventional and novel agents including bortezomib. The lymphoma cell line DOHH2 (*t(14;18)*), U266 (myeloma), K562 (CML) and primary CLL cells were used in this study with HL60 (AML) as a control. Cells were seeded in 96 well plates and treated with a variety of drugs alone or in combination (cytarabine, fludarabine, doxorubicin, daunorubicin, etoposide, melphalan, bortezomib) at multiple concentrations. Measurements were taken at 5-min intervals for up to 48 hours and analysed using KORsoft software to generate apoptotic response curves, which were compared to conventional techniques. The KOR assay can show maximum sensitivity ( $S_{max}$ ) to cytotoxics. DOHH2 was found to be dose responsive to four of the drugs used. There was no effect from fludarabine, but the addition of bortezomib increased  $S_{max}$  when in combination with etoposide. Parallel flow cytometric analysis using Annexin V and PI showed similar results to those from the KOR assay, confirming the assessment of apoptosis to be valid. Primary CLL samples were cultured with and without IL4 and run in the KOR assay with cytotoxics including bortezomib. Culture with IL4 alone gave good growth characteristics and revealed the combination of etoposide and bortezomib to provide the best induction of apoptosis. The combination of bortezomib and etoposide appears effective for lymphoma. Such approaches can accelerate the development of clinical trials.

## Poster Presentations: Myeloid Malignancy

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**Prognostic relevance of circulating matrix metalloproteinase-2 in acute myeloid leukemia****S Aref\*, E Osman†, S Mansy†, N Omer†, E Azmy‡ and T Goda§***\*Department of Haematology, Mansoura Faculty of Medicine,**†Mansour Faculty of Science, Mansoura University,**‡Haematology-Oncology Unit, Mansoura Faculty of Medicine,**§Department of Internal Medicine, Mansoura Faculty of Medicine*

matrix metalloproteinase (MMPs) were postulated to have important implication in progression and invasiveness of many malignant disorders. On the other hand the biological role of MMP-2 in acute myeloid leukemia is not fully clear.

Serum MMP-2 concentration levels was determined in 37 AML patients pretreatment and for 20 AML patients after induction chemotherapy using enzyme linked immuno-sorbent assay and were compared to thier levels in normal healthy control group ( $n = 10$ ).

The pretreatment sMMP-2 levels were significantly lower as compared to post induction level ( $P = 0.000$ ) and control levels ( $P = 0.007$ ). Pretreatment sMMP-2 levels were not significantly correlated to FAB subtypes, Peripheral blood blast cell counts, Peripheral WBCs counts, BM blast cell counts, and blast cell distribution ratio. Patients who had high pretreatment levels of sMMP-2 had particularly poor outcome. High sMMP-2 had an independent adverbs influence on survival, it was entered as a factor into multivariate analysis together with other prognostic factors (relative risk 9.0, confidence interval 0.94–86.0,  $P = 0.01$ ).

**Conclusion:** High pretreatment levels of sMMP-2 are associated with poor survival in patients with AML



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**Rapid screening for gene mutations in AML patients using pyrosequencing****M Lazenby, AF Gilkes, V Walsh, AK Burnett and KI Mills***Department of Haematology, Cardiff University, Cardiff, UK*

Multiple genetic alterations are usually required for the development of acute myeloid leukaemia. In addition to the major cytogenetic abnormalities such as translocations  $t(15;17)$ ,  $t(8;21)$  and  $inv(16)$  several genes are commonly mutated including FLT3, NPM, MLL and c-kit. Some of these secondary mutations have been shown to add prognostic significance to the cytogenetic based risk groups. Therefore, identification of these mutations at diagnosis is important not only for assigning possible prognostic groups, but also for use as a means of monitoring minimal residual disease. The development of novel therapeutic agents, such as FLT3 inhibitors, further reinforces the need for early identification of mutation status to allow the inhibitors to be used on the appropriate patient group.

We have developed pyrosequencing assays for the rapid detection of FLT3-TKD, npm1, and c-KIT mutations. Pyrosequencing assays have advantages over standard PCR/restriction digest techniques in that they are faster, allow different mutations within a region on the target gene to be analysed from a single PCR, and give the precise sequence of the mutation without the need for further analysis. An estimate of the size of clone with the mutation may also be obtained. Ninety-six wells can be processed post-PCR within 90 min producing multiple gene mutation information for multiple patient samples.

These assays have been used prospectively to analyse RNA isolated from AML patient diagnostic samples for the presence of FLT-TKD, NPM and c-kit mutations. Pyrosequencing is unsuitable for detection of FLT3-ITD mutations as the start site and length of the duplication is highly variable. FLT3-ITD results were obtained using a different technique. To date, 130 AML samples have been prospectively analysed; 22% have FLT3-ITD mutations with pyrosequencing detecting 3.1% with FLT-TKD, 16.9% with NPM1 and 3.1% with c-kit mutations.

The rapid and versatile methodology of pyrosequencing will add to a leukaemia diagnosis repertoire.

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**A molecular classification of leukaemia reveals MDS as a disease continuum with non-leukaemia and AML sub groups****KI Mills<sup>\*,||</sup>, T Haferlach<sup>†,||</sup>, JM Hernandez<sup>‡,||</sup>, W-K Hofmann<sup>§,||</sup>, A Kohlmann<sup>||</sup>, M Williams<sup>||</sup> and L Wiczorek<sup>\*</sup>**<sup>\*</sup>*Department of Haematology, Cardiff University, Cardiff, UK,*<sup>†</sup>*MLL, Munich, Germany, ‡Hematologia, Salamanca, Spain,*<sup>§</sup>*Charite, Berlin, Germany, ||Roche Molecular Systems,**Pleasanton, CA, USA, ||MILE Study on behalf of European**LeukemiaNET (ELN)*

Recently, the MILE (Microarray Innovations in LEukemia) study has analysed ~2000 expression profiles of 16 acute and chronic leukaemia subclasses, MDS, and non-leukaemia as control group in 11 centres (ELN: seven, USA: three, Singapore: one) and compared the microarray classification accuracy, to routine diagnostic workup. The overall cross-validation accuracy was very high for the leukaemia subclasses: ~96%.

However, only 49.1% of the 173 MDS samples included in the study were correctly called as MDS from their underlying gene expression profiles. The remainder were approximately equally split between a call of 'non-leukaemia' (24%) and 'AML' (24.6%). Our

analysis showed that neither study centre nor age were a factor in differentiating between 'MDS', 'MDS with an AML-like signature' or 'MDS with a non-leukaemia like signature'. WHO classification was highly correlated with the microarray classification result; specifically RAEB (I or II) was associated with 'AML' call ( $P < 0.0001$ ) whilst, RA/RARS was highly correlated with 'MDS' or 'non-leukaemia' calls. Furthermore, IPSS was significantly correlated with call ( $P > 0.0001$ ): 65% of patients with an IPSS score of Int-2 or above were classified as 'AML'. Individually, the blast, karyotype and cytopenia contributions were highly significant ( $P < 0.0001$ ,  $< 0.013$  and  $< 0.0001$  respectively) when comparing 'MDS like AML', 'MDS' and 'MDS like non-leukaemia' samples. Survival data (available for 122 of the diagnosed MDS patients) showed that MDS patients called 'MDS like AML' had a trend towards shorter survival ( $2P = 0.2$ ) than those called 'MDS' or 'MDS like non-leukaemia'. The mapped molecular pathways and functions between these sub-groups may give an indication of the molecular steps involved in disease evolution and lead to a molecular redefinition of MDS.

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**Embryonic gene (Oct-4) expression in normal adult bone marrow CD133 + CD34 + stem cells****X Zhai, JA Goodacre and HT Hassan***Lancashire School of Postgraduate Medicine, University of Central Lancashire, Preston, UK*

Human CD133 stem cell antigen is expressed in several normal adult stem cells including endothelial, basal epidermal, prostate epithelial, haematopoietic, mesenchymal and neural cells. In adult bone marrow, 90% of human haematopoietic stem cells co-express CD133 and CD34 and are mostly in quiescent phase of cell cycle.

Recently, the expression of binding transcription factor Oct-4, a marker of embryonic pluripotent stem cells was reported in several adult human non-haematopoietic stem cells including epithelial and basal epidermal cells ([2005] Carcinogenesis 26: 495–502). The present study was performed to evaluate similar Oct-4 expression in normal adult haematopoietic stem cells. Immune-fluorescence staining using Anti-Oct-4 monoclonal antibodies of isolated bone marrow CD133 + CD34 + stem cells from healthy adults revealed the expression of Oct-4 in two thirds of these haematopoietic marrow stem cells as shown in the table.

The present results demonstrate substantial expression of embryonic stem cell marker Oct-4 in human haematopoietic stem cells in adult bone marrow. We recently reported similar expression of Oct-4 in human CD133 + CD34 + AML cells ([2006] Haematologica 91(s1): 426). Oct-4 expression in both normal and leukaemia CD133 + CD34 + stem cells in adult human bone marrow suggests their embryonic stemness renders them the most likely target for development of AML. Immunophenotyping of marrow samples from de novo AML patients revealed strong presence of CD133 + CD34 + stem cells (>20%) in two thirds of AML cases ([2001] Haematologica 86:154–161) and their capability of forming leukaemic CFU *in vitro* ([2001] Cytotherapy 3: 449–459) further supporting the role of these stem cells in the propagation of AML.

Oct-4 Expression	Percentage	Bright	Dim	Negative
in Human Adult Bone Marrow CD133 + CD34 + Stem Cells	Median	33.1%	37.9%	29.0%
	Range	19.5–45.4	25.0–46.5	16.7–43.5

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### Are we NICE to chronic myeloid leukaemia patients in the northern cancer network?

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Targeted therapy has significantly altered the prognosis for patients with CML. Technology Appraisal Guidance documents published by NICE in September 2002 and October 2003 recommended the use of imatinib mesylate for chronic phase CML patients intolerant of/ resistant to interferon, and for newly diagnosed patients respectively. Patient groups have questioned the access of CML patients to imatinib and specialist haematology care. A retrospective study of all patients in the Northern Cancer Network diagnosed with CML has allowed us to review epidemiology of the disease, local adherence to these national guidelines and quality of care.

All new Philadelphia chromosome positive/Philadelphia chromosome negative bcr/abl positive patients were identified for the period January 1996–December 2005 from records held at the single regional cytogenetics laboratory. pH +ve ALL patients were excluded. Available case notes were reviewed. 166 new patients with CML were diagnosed giving a crude annual incidence of 0.8 per 100,000 in our population of 2.1 million. 148 case notes were available for further evaluation.

146 patients were managed by consultant haematologists. One elderly patient is monitored by FBC sent from her nursing home. One patient declines treatment. 56 patients have died. Of the surviving 92, two have changed areas and current treatment details are unavailable. 84 were treated in accordance with NICE guidelines. Six are not currently treated according to NICE guidelines (4 of these were imatinib intolerant). Details of imatinib response will be presented. In the five year period 1996–2000, 12 allogeneic transplants were performed compared to 2 in the period 2001–5.

This population based study confirms a lower incidence of CML in the Northern Cancer Network compared to published data. We find no evidence of restricted access to specialist care or imatinib. Our practice reflects the global decrease in transplantation in the post imatinib era.

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### Serum selenium at presentation may predict disease outcome in acute myeloid leukaemia (AML)

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We have previously shown that patients with aggressive lymphoma and low serum selenium at presentation have a less favourable response rate and overall survival. We have extended these observations to AML.

Inductively coupled mass spectrometry was used to measure serum selenium concentration from 163 adults, 16–60 years, with newly diagnosed AML. Mean serum selenium was 68.2 µg/l. Selenium concentration showed a negative association with white cell count ( $r = -0.28$ ,  $P = 0.0003$ ) and positive association with albumin

( $r = 0.33$ ,  $P < 0.0001$ ); no relationship was seen with gender, age or karyotype.

Patients were divided by their serum selenium into 'normal' (within UK reference range, 70.2 – 58 µg/l) or 'low' (below range), with 80(49%) and 83(51%) patients in each group respectively. Of those evaluable, remission after induction therapy was achieved in 57 (71%) patients in the normal group and 48 (58%) in the low group. Thirteen (16%) and 19 (23%) patients in each respective group were refractory to treatment, with 6 (8%) and 13 (16%) deaths. A chi-squared test on induction data showed a better outcome for patients with normal selenium ( $P = 0.04$ ). Overall survival was better in the group with normal selenium ( $P = 0.08$ ). Univariate Cox analysis gave a hazard ratio of 1.43 (95% CI 0.96–2.13) for patients in the low selenium group. There was no similar relationship between selenium at presentation and progression free survival.

In multivariate Cox analysis, selenium was not predictive of survival once the more significant factors of albumin and karyotype were added to the model. However, an interaction term between selenium and albumin was added ( $P = 0.05$ ), suggesting that changes in albumin level have a larger effect on survival for patients with normal selenium. The longest survival was predicted for patients with both high albumin and normal selenium at presentation.

These data support the hypothesis that serum selenium at presentation may predict outcome in AML patients.

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### Safety and feasibility of home delivery of low dose cytarabine by continuous subcutaneous infusion in acute myeloid leukaemia

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It is reported that administration of Cytarabine by continuous intravenous infusion is more effective than giving bolus doses (intravenously or subcutaneously) since Cytarabine is quickly deaminated in the liver to Uracil Arabinoside, which is non active. The Baxter infusion balloon-pump is ideal for home treatment, but there is no experience of continuous subcutaneous delivery of Cytarabine by this system, keeping the pump close to the body. Cytarabine is stable in this infusor at 25degC, but there are no stability data when Cytarabine is maintained at near-body temperatures.

We prepared 2 Baxter 7-day Infusors with Cytarabine 140 mg in 95 ml of 0.9% normal saline (1.47 mg/ml). One was tested at time 0 and then discarded, and the other was incubated at 35°C and tested after 7 days. Samples were analysed using High Powered Liquid Chromatography (HPLC) (Applied Analysis Ltd). Once the stability of the Cytarabine was established, patients with AML wanting non-intensive chemotherapy were offered Cytarabine 20 mg daily by continuous pump subcutaneously as an outpatient for 7 to 21 days.

The concentration of Cytarabine in the infusor fell from 95% at time 0 to 93% at 7 days and the pH fell from 7 to 5.6. HPLC showed the only degradation product was Uracil Arabinoside. There was no solution discolouration nor crystal deposition.

Six patients were treated with 26, 7-day Baxter-infusors. All patients found the portable pump acceptable. A subcutaneous lump formed under the needle after 4–5 days causing slight discomfort in some. Most patients chose to have the needle re-sited, but some continued for 7 days without discomfort. One patient with thrombocytopenia experienced local bleeding, and one developed local infection when neutropenic.

Our results show that Cytarabine is stable for 7 days in the Baxter balloon infusor even at close to body temperature. Patient acceptability was good with few practical difficulties and few hospital attendances.

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### Poor response to imatinib therapy in chronic myeloid leukaemia expressing variant BCR-ABL transcripts

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In CML, imatinib produces complete cytogenetic remission (CCR) in approximately 80% of newly diagnosed cases, in about 50% of chronic phase patients who have failed interferon, and in 10–20% of patients with more advanced disease. At least 98% of CML patients express either one or both of e13a2 (b2a2) or e14a2 (b3a2) BCR-ABL fusion transcripts arising from breakpoints in the major breakpoint cluster region of BCR. Variant transcripts arising from outside the major breakpoint cluster region are rare. These variant transcripts are increasingly being reported, however, their associated disease phenotype remains unclear. There are no data currently available on the efficacy of imatinib in patients with variant transcripts.

From October 2000, 221 cases of CML have been molecularly screened at our institution. 217 expressed one or both of the major breakpoint cluster region transcripts e13a2 or e14a2.

Here, we report the effect of imatinib in the remaining four cases, each expressing a variant transcript: e13a3, e19a2 (two patients) and e6a2. Two of the patients presented in chronic phase, (treated with 400 mg daily), one in blast crisis and the other in accelerated phase (each treated with 600 mg daily). Three cases died within 6 months of commencing imatinib therapy without achieving CCR, and the remaining case died after 4 years of therapy, again without achieving CCR. All patients died of disease progression.

As a result of varying breakpoints, the three dimensional structure of the BCR-ABL fusion protein may differ from that of 'classic' transcripts. This may alter the capacity for tyrosine kinase drug interaction. To our knowledge, this is the first report of the effects of imatinib in patients with variant BCR-ABL transcripts. At present, we suggest caution in extrapolating the excellent results of imatinib therapy in major breakpoint CML to patients with variant transcripts.

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### Empirically diagnosed *Aspergillus* infection and the curtailment of chemotherapy for AML. Five years experience in a single Institution

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To assess the impact of *Aspergillus* infection on treatment for AML, we studied the records of patients treated with intensive chemotherapy over a 5-year period. Diagnosing fungal infection according to the EORTC/IFICG (2002) stratification is impractical in a District General Hospital. We therefore classified our patients with suspected *Aspergillus* infection into three groups: refractory fever only, refractory fever + radiological evidence of lung infiltration and refractory fever + microbiological evidence. All patients assessed (26) were treated with curative intent in NCRI protocols. Their ages ranged from seventeen to 79. Seventeen patients (65%) failed to complete the specified number of chemotherapy cycles. In three this was due to refractory disease and in two due to patient choice. The remainder had their treatment curtailed due to complications that

were considered life-threatening. One of these had isolated chemotherapy toxicity, four had severe microbiologically proven bacterial septicaemias, one had severe candida septicaemia and six (23% of our total group) had suspected *Aspergillus* infection. Of these six, microbiological evidence was only available in two. One patient had radiological evidence and three had refractory fever only. All six had received intensive anti-microbial therapy with liposome-encapsulated amphotericin but were considered too frail to receive further consolidation chemotherapy. Twelve of the 17 patients who failed to complete their treatment have died, five survive in remission with a median follow up of 3 years. Nine patients completed all cycles of their scheduled treatment, six of these have died and three are in remission. In this group, four were suspected of having *aspergillus*, three on radiological evidence and one because of refractory fever only. In this small series, empirically diagnosed *Aspergillus* infections were more common than is quoted in the literature. They resulted in curtailment of treatment in almost a quarter of the patients but this does not seem to have had an adverse impact on outcomes.

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### Successful cytogenetic remission following the use of dasatinib in a patient with BCR-ABL positive chronic myeloid leukemia who relapsed following nilotinib therapy

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A 57 year old female with chronic myeloid leukaemia went into blast crisis at 10 months after achieving complete cytogenetic remission with glivec 400 mg. At the time of relapse her BCR-ABL: ABL ratio increased to 155% from a residual level of 11% with reappearance of original cytogenetic rearrangement between chromosome 9q, 12q and 22q together with monosomy seven indicating cytogenetic clonal evolution. She showed a brief response to two courses of daunorubicin and cytosine arabinoside but soon relapsed. She was now commenced on nilotinib (AMN107) at a dose of 400 mg twice daily and achieved major cytogenetic response (2% abnormal metaphase cells), and with a significant reduction in the BCR-ABL:ABL ratio (31%) that was sustained for only six months. She was now commenced on dasatinib 50 mgs twice daily and achieved complete cytogenetic and major molecular response (>3 log reduction in BCR-ABL transcripts) that is maintained at six months. Mutation analysis detected two mutations at the time of first blast crisis (F359V and H396P) and a new mutation was found (Y253H) following a loss of response to nilotinib. As her responses to treatment were not sustained it is likely that she developed mutations at the time of each relapses (further samples under analysis), however it is also possible that other additional genetic changes were driving some of the relapses, e.g. related to the monosomy 7. Her current molecular response to dasatinib suggests effectiveness of this drug against her nilotinib resistant clones. More follow up is however needed to see how long this response is sustained. This case highlights the fact that Dasatinib is a highly potent tyrosine kinase inhibitor, which can be effective against imatinib and nilotinib resistant clones. The response to 1st and 2nd generation tyrosine kinase inhibitors in sequence may suggest using these agents in combination after detailed mutational analysis.

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### Artificial neural network analysis identifies novel patterns of *HOX* gene expression in prognostic subgroups of acute myeloid leukaemia

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The acute myeloid leukaemias (AMLs) are a heterogeneous group of haematological malignancies with diverse clinical outcomes. Pre-treatment karyotype analysis identifies biologically distinct subgroups and is currently used as a predictor of response to induction chemotherapy and risk of relapse. Cases may be stratified into relatively favourable, intermediate and adverse prognostic subgroups. *HOX* genes encode master transcription factors which regulate key developmental processes. Humans have 39 *HOX* genes and multiple lines of evidence implicate their deregulated expression in the pathogenesis of AML. Drabkin *et al.* (Leukemia 2002; 16: 186–95) have reported that AMLs with a relatively favourable prognostic karyotype are associated with low levels of *HOX* gene expression whereas AMLs with an adverse prognostic karyotype have higher levels of expression. To further characterise *HOX* gene expression in cytogenetic prognostic subgroups we determined the expression profiles of 26 *HOX* genes by real-time quantitative PCR (Q-PCR) in diagnostic samples, representative of the three prognostic subgroups, from 25 patients with de novo AML. Profiles were then analyzed using Artificial Neural Network based computational approaches to identify a subset of *HOX* genes which could discriminate between prognostic subgroups in a predictive fashion. The relatively favourable prognosis subgroup was primarily defined by down-regulation of *HOXA5* and up-regulation of *HOXC4*. The expression of six and eleven *HOX* genes were required to define membership of the intermediate prognosis and adverse prognosis subgroups respectively, reflecting both the molecular complexity of these subgroups and the small sample size. The results show that Artificial Neural Network based computational approaches are capable of further characterising *HOX* gene expression within AML prognostic subgroups as determined by presenting karyotype and that measuring the expression levels of a small number of *HOX* genes at diagnosis can provide useful clinical information in cases where karyotype analysis has been unsuccessful.

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### The quality and utility of stem cells cryopreserved in chronic phase chronic myeloid leukaemia (CML): is routine leucopheresis still cost effective?

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As a result of successful targeted therapy such as Imatinib and improvements in the outcome of allogeneic transplantation in CML the practice of autologous SCT is declining.

We performed a retrospective audit of patients with chronic phase CML who underwent leucodepletion and/or stem cell storage in Bristol over an eight year period from 1998 to 2006. The aims of the

audit were to assess the criteria for referral and the quality of stem cells collected with respect to CD34 + cell count and engraftment.

A total of 56 patients (19 women, 37 men) with chronic phase CML were referred for apheresis procedures. Fifty-four patients also required stem cell collection and storage. Two patients underwent cytoreduction without stem cell storage because one patient was not a candidate for transplantation and the other had imatinib resistant CML.

The mean WBC count of the whole group was 225.32 (range 3.7 – 814)  $\times 10^9/l$ . Eight patients had symptoms of leukostasis with a mean WBC 403 (range 216–814)  $\times 10^9/l$ , and required between one and five procedures for effective cytoreduction. There were no reported significant complications. The first apheresis procedure led to an average reduction in white cell count of 30%. The mean dose of CD34 + cells cryopreserved was 66.95  $\times 10^6/kg$  (range 4.92–210.62). None of the stored autologous stem cells were infused. Twelve patients received allogeneic stem cell transplants.

This audit demonstrated that adequate stem cell doses were collected at presentation but that in eight years none of these collections were actually used. This calls into question the cost effectiveness of the common practice of cryopreservation of stem cells at presentation. Designing the audit also highlighted the lack of guidelines for cytoreduction in chronic phase CML. All patients with symptoms of leukostasis had a WBC greater than 200  $\times 10^9/l$ .

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### Central nervous system lymphoid blast crisis in a patient with haematological remission of chronic myeloid leukaemia (CML) on dasatinib

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Dasatinib(BMS-354825) is now available to treat imatinib-resistant CML. A preliminary study of K562 CML cell lines implanted intracranially in SCID-beige mice showed growth stasis of these cells during dasatinib therapy and increased lifespan compared to controls, suggesting that dasatinib may have therapeutic advantages over imatinib in management of intracranial CML(Wild *et al.*, [2004] *Blood*, 104 11). We report CNS lymphoid blast crisis developing in a patient with haematological response on dasatinib.

A 20-year-old male with chronic phase (CP) CML received hydroxyurea, interferon, then imatinib three years later. Haematological response, without cytogenetic response, occurred on imatinib doses to 600 mg/day. Tibial biopsy for tibial pain developing after two years on imatinib revealed lymphoid blasts (CD10 +, TdT +), also present in bone marrow (BM > 30% lymphoblasts); at this time CNS was clear. After two courses of fludarabine/cytarabine/G-CSF/ idarubicin (FLAG-Ida), second CP occurred. Due to cytopenias and gastrointestinal disturbance methotrexate/6-mercaptopurinemaintenance was stopped. White cell and platelet counts increased rapidly, with 7% blasts by BM immunophenotyping. He then commenced dasatinib 140 mg/day without CNS prophylaxis. Three months later, on dasatinib, he developed headaches, ataxia and burning foot pains. CT brain was normal. CSF showed 95% B-ALL blasts. The blood count was normal, and BM showed CML in CP, with 0.5% TdT + cells. Thus CNS blast crisis developed despite haematological response to dasatinib.

In a phase II trial of dasatinib, one lymphoid blast phase CML and five Ph + ALL with documented CNS leukaemia were treated with

dasatinib; 4/6 attained complete cytogenetic response, five had documented CSF resolution of CNS leukaemia with dasatinib monotherapy, and one had CNS disease resolution with dasatinib/intrathecal cytarabine. In our case CNS disease appeared or was not suppressed by dasatinib; this may have been due to development of

resistant mutations. CNS prophylaxis appears necessary in CML lymphoid blast crisis treated with dasatinib. Whether concomitant dasatinib with CNS prophylaxis increases CNS toxicity will be determined by more extensive usage of dasatinib.

## Poster Presentations: Nursing

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### **A visual guide to the administration of blood components**

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The administration of blood components can be a potentially risky if not fatal process. These risks can be present in areas where administration of blood components are given frequently as staff can become blasé or complacent about the procedure. In areas where blood products are seldom given staff will arguably need extra support. To address all areas where these components are given a simple flow chart indicating best practice as recommended by the British Committee for Standards in Haematology has been established.

This algorithm is in a simple format and clearly shows how different components should be administered. It is user friendly and as appropriate to senior haematology nursing staff as it is to junior/student nurses and those unfamiliar to haematology.

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### **Role of the haematology specialist nurse in the management of patients with suspected myeloproliferative disorders**

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The Royal Bournemouth Hospital is a general hospital serving a population of 350 000 people. An audit was carried out of patients who were referred by their GPs for the investigation of a raised haemoglobin, haematocrit or platelet count between August 2002 and March 2006.

Over a 3-year 7-month period 322 patients underwent extensive investigations including ultrasonography, erythropoietin levels, JAK testing, bone marrow analysis and red cell masses. Two hundred and sixty-four patients were diagnosed as having a non-myeloproliferative condition. One hundred and five of these patients with non-primary haematological conditions are still being seen regularly in the haematology department.

From these 322 referrals:

Nineteen patients were diagnosed as polycythaemia vera,

Thirty-nine were diagnosed as essential thrombocythaemia.

Seventy-seven were transient polycythaemia/thrombocythaemia

Eighty-five were secondary/reactive polycythaemia/thrombocythaemia

Seventy-one were idiopathic polycythaemia/thrombocythaemia

From this audit it could be seen that Consultant Haematologists are spending a proportion of their clinic appointment slots dealing with the investigation of potential myeloproliferative disorders. Once diagnosed patients continue to return to clinic for management or treatment of their condition.

Funding was made available for the post of a Haematology Nurse Specialist for the out-patient department and her role consists of the following:

- Regular management of stable MPD patients treated with hydroxycarbamide/venesection with emphasis on good patient information at diagnosis and self care strategies in order to manage side effects of treatment.

- Streamlining of investigations for referred polycythaemia/thrombocythaemia patients. This includes ensuring results of basic investigations are available to the haematologist at the first visit.

- Management of secondary polycythaemia with emphasis on encouraging and giving information about lifestyle changes.

It is hoped that this new service will provide a cost-effective alternative for the Trust that will allow Consultant Haematologists to deal with more complex cases. Patients will receive a timely and quality service with emphasis on helping them to cope with their condition by giving information and in some cases trying to reduce its severity by encouraging a change to lifestyle.

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### **Sickle cell vaso-occlusive attack and integrated care pathways**

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There is a population of less than 30 people suffering with sickle cell disorder in the South Wales area. Many weeks or months may elapse before the medical team in the Cardiff and Vale trust care for a patient suffering with a vaso occlusive attack. Consequently the care for this client group has been inconsistent.

In early 2004 a multidisciplinary team was assembled to develop an integrated care pathway based on the British Society of Haematologists guidelines for the patient with a vaso occlusive attack. The document was implemented in August 2004 and an Audit performed 24 months later.

The purpose of the Audit was to ascertain if the pathway was used and if it was used properly, also to see if the goals set out (such as pain relief given within 30 min) in the pathway were met.

The findings were that out of 18 admissions the pathway was used 14 times, with only 10 of the documents being completed adequately. Two patients received pain control within 30 min but 10 having pain assessments performed. It was found that only nurses filled out the care pathway after the 1st day. In conclusion it was found staff need to be consulted as to why the document is not being widely used and that more education is needed with medical and nursing staff.

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### **Audit of the appropriateness of referrals to the nurse/pharmacist-led hydroxycarbamide service at the RUH**

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Hydroxycarbamide is used to treat patients with essential thrombocythaemia, polycythaemia rubra vera and other myeloproliferative disorders. Historically, all patients attended the RUH Haematology Clinic to have their FBC checked and Hydroxycarbamide prescribed, with little or no dosage change. The patients often travelled long

distances, waited in clinic to be seen, and then had a brief appointment with a clinician. A Nurse/Pharmacist-led service has been set up at the RUH so that patients who are stable on Hydroxycarbamide are referred to the service by the clinician and have blood tests at their GP surgery at specified time intervals. The results are reviewed by the Specialist Nurse or Pharmacist and the patient told by phone whether they need to change their Hydroxycarbamide dose. The aim of the audit was to ascertain whether patients were being appropriately referred to the service. Enrolling as many patients as possible onto the Nurse/Pharmacist-led service may result in a reduction in an over-booked Haematology clinic.

## **Poster Presentations: Paediatrics**

205

### **Childhood extramedullary acute lymphoblastic leukaemia: how should it be managed?**

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Extramedullary acute lymphoblastic leukaemia (ALL) with no bone marrow disease is extremely rare at first presentation of disease. It can occur in many different sites and is referred most frequently to surgeons. Incorrect diagnosis of lymphoma can be made if thorough investigation is not carried out. There are no established guidelines for its treatment. Intensive multi-agent therapy is recommended despite the frequent small amount of localised disease. Treatment is successful in the majority of patients with overall survival similar to ALL presenting with bone marrow disease.

We present four cases of precursor B-cell ALL that have presented to our institution over a period of six years. Ages at presentation ranged from 3 to 12 years. Sites of involvement include skin, subcutaneous soft tissue and a lymph node. Morphology in each case revealed the presence of intermediate or large lymphoblasts with irregular nuclei and several nucleoli. Immunohistochemistry showed cells of B-cell lineage and Tdt positivity consistent with precursor B-cell ALL. In all cases further investigation revealed no other sites of disease and no bone marrow involvement.

Each patient was treated with multi-agent chemotherapy using the current national ALL treatment protocol at time of diagnosis. Response to therapy in each case was assessed clinically and wider leukaemia burden assessed as directed in their treatment protocol. Two patients continue on treatment. In all cases response, determined clinically because of their localised disease, has been complete.

Extramedullary ALL is rare and presentation pattern is variable. Diagnosis can be difficult but it can be successfully treated with different regimens and these are discussed. The expectation is that success of therapy will give overall survival similar to patients with disease only affecting bone marrow. There are no specific guidelines for management of extramedullary ALL in children. Consensus for management and data collection for this rare group of patients is needed.

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### **Recombinant activated factor VII as treatment for children with severe congenital platelet disorders. Experience of a single paediatric haemophilia centre**

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Bleeding episodes in children with severe congenital platelet disorders are associated with significant morbidity and mortality. Historically, platelet transfusions have been used to achieve haemostasis but recent concerns about transfusion transmitted infection and the risk of platelet alloimmunisation have led to the proposed use of recombinant activated factor VII (rFVIIa) (NovoSeven; Novo Nordisk) as an alternative treatment.

We assessed the treatment of 63 episodes of bleeding in two children with Bernard Soulier Syndrome (BSS) and three children with Glanzmann's Thrombasthenia (GT) treated between 2000 and 2006. Bleeds were classified as mild (57) or severe (6) depending on whether the patients were haemodynamically compromised and/or had a drop in haemoglobin  $> 2$  g/l from their baseline. In all bleeding episodes rFVIIa was given as first line therapy in a dose of 90 µg/kg, repeated after 2 hours if no initial response had been obtained. Efficacy of rFVIIa in achieving haemostasis was effective if bleeding had stopped at less than or equal to 6 hours from presentation.

Overall, 73% of bleeding episodes responded to rFVIIa. rFVIIa treatment was ineffective in patients presenting with severe bleeding episodes, all of whom required platelet transfusions to achieve haemostasis. Twenty-seven out of thirty-six (75%) of mild bleeding episodes in GT patients responded to rFVIIa. Of these, 78% responded to a single dose. Nineteen out of twenty-one (90%) of mild bleeding episodes in BSS patients responded to rFVIIa; 79% of these responding to a single dose.

This study confirms that rFVIIa is a useful alternative to platelet transfusion as a treatment for mild bleeding in children with BSS or GT. Perhaps surprisingly, it would appear to be more effective patients with BSS rather than GT, though this observation will require further evaluation. Our experience would suggest platelet transfusions should remain first line treatment for all severe bleeding episodes in BSS and GT.

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### Nocturnal hypoxemia and intracranial vessel turbulence on magnetic resonance angiography in children with sickle cell disease

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Nocturnal hypoxemia predicts CNS events in sickle cell disease (SCD). Underlying mechanisms are poorly understood but may include hypoxemia-mediated activation of inflammatory cells resulting in increased cellular adhesion or an interaction with haemolysis. In patients, the severity of hypoxemia might be associated with the degree of increased turbulence in the terminal internal carotid, middle and anterior cerebral arteries, which has been shown to correlate with the degree of distal MR perfusion abnormality. Time-of-flight magnetic resonance angiography (MRA) was performed in 56 patients with SCD (30 boys; median age 8 years, range 2–16 years). Each prospectively had a sleep study, MRA and reticulocytes count. Turbulence on MRA was graded as 0 (none), or as increasing severity grades 1–3 by two radiologists blinded to the data.

There was a significant difference in mean overnight SpO<sub>2</sub> for those with different degrees of turbulence ( $P = 0.003$ , Kruskal–Wallis). Post-hoc analysis revealed a significantly lower mean SpO<sub>2</sub> in patients with grade 2 ( $n = 13$ ; median 94.7%, range 85–97.8%) and grade 3 ( $n = 14$ ; median 93.6%, range 87–97.5%) turbulence than in those with no vascular disease ( $n = 21$ ; median 96.5%, range 90.2–99.5%) ( $P = 0.008$  and  $0.021$  respectively). There was no difference between the mean overnight SpO<sub>2</sub> in those with grade 1 turbulence ( $n = 8$ ; median 96.1%, range 92.4–98.3%) and any of the other groups or between those with grade 2 and 3 turbulence. Reticulocyte count was significantly higher in those with grade 3 turbulence than in those with no turbulence. In a binary logistic model (grades 0 or 1 turbulence vs grade 2 or 3), mean overnight SpO<sub>2</sub> ( $P = 0.0001$ ) and reticulocyte count ( $P = 0.01$ ) were independently associated with turbulence.

Our results suggest an association between the degree of nocturnal hypoxemia and severity of intracranial vasculopathy in

children with SCD. Correcting potential causes of hypoxemia may substantially reduce the risk of stroke in this population.

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### Transition services in sickle cell disease: patients' fears and expectations

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Sickle cell is due a single amino acid substitution of the beta-chain of haemoglobin of valine for glutamic acid in position six. The central phenomenon is the tendency for paracrystal formation of deoxygenated haemoglobin and subsequent vaso-occlusion and is responsible for pain, end-organ damage such as stroke, renal disorders, delayed puberty and psychosocial development.

The provision of transition services presents particular challenges in relation to the development of self independence, adaptation, and self-determination. These issues are more profound sickle cell disease as a chronic condition and the unpredictable nature of the pain with the feeling of loss of control.

We set out to determine the views of teenagers on their expectation for service provision and their fears for the future. A semi-structured questionnaire jointly developed by doctors, psychologists, nurses and teenagers was initially piloted on a variety of children and then administered to 41 adolescent sickle cell patients attending Evelina Children Hospital.

The following suggestions for service improvement were made: teenage books and magazines and computers games, social and IT information areas. A significant portion (55%) of the patients wanted to talk about personal issues such as sex, alcohol and drugs. Up to 41% suggested setting up of a social group or camp so they can meet with their peers with chronic illness and just over 50% wish to be seen alone without their parents in clinic.

Their biggest fear was 'fear of death' or dying at a young age (30%) and pain (15%), stroke (7%), or passing the sickle cell gene to their children.

Conclusions: This highlights the need for confidentiality, development of independence for patients to be seen on their own in clinic. The main cause of anxiety is the fear of death and dying at young age, or passing sickle cell to their children.

## Poster Presentations: Red Cell Disorders

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### Circulating DNA as a biomarker in sickle cell disease

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Free circulating DNA has been shown to be present in the plasma of healthy subjects and elevated in conditions characterised by increased cell death, such as cancer and physical trauma. In sickle cell disease (SCD) an increased cell turnover can be expected through recurrent episodes of vaso-occlusion and inflammation, leading to cell death and organ damage. We proposed that circulating DNA

levels would be higher in patients with SCD, that these elevations would increase with acute crises and that the extent of increase may serve as a marker of disease severity. Plasma samples were collected from patients with SCD attending the specialist clinic at King's College Hospital. Over a 2-year period, 154 patients (105 HbSS, 46 HbSC, 3 HbS/betao thalassaemia) provided samples at each visit to the specialist clinic and also during an acute crisis, as defined by hospital admission for sickle-related pain, giving a total of 418 samples. The ethnically-matched control population consisted of 53 non-SCD individuals. Plasma DNA concentration was measured by real-time quantitative PCR using a probe specific for the beta-globin gene. There was no significant difference in plasma DNA levels between controls and SCD patients during steady state. However, mean plasma DNA levels for sickle patients during steady state, and those in crisis were highly significantly different ( $P < 0.0001$ ). Circulating DNA levels correlated with CRP levels in the SS/Sbetao ( $r = 0.24$ ,  $P < 0.005$ ) and SC groups ( $r = 0.31$ ,  $p < 0.05$ ) but not in the

controls. DNA levels correlated with WBC in the SS/Sbetao group only ( $r = 0.25$ ,  $P < 0.05$ ). Our preliminary studies showed that, unexpectedly, circulating DNA levels were not elevated in steady state SCD despite the ongoing inflammatory state. However, DNA concentration may be a reliable biomarker in SCD crisis. We are currently carrying out longitudinal studies to explore the value of serial measurement of plasma DNA levels and their association with organ damage in SCD.

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### The interaction of UGT1A, HO1 and alpha-thalassaemia variants with bilirubin levels and gallstones in sickle cell disease

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Chronic hyperbilirubinaemia is common in patients with sickle cell disease (SCD), frequently resulting in gallstone formation. It is attributed to haemolysis that exceeds the conjugating capacity of the hepatic UDP-glucuronosyltransferase (UGT1A) enzyme. Previous studies have shown that genetic variants ([TA]<sub>n</sub> repeats) in the UGT1A gene promoter region have a major influence on bilirubin levels and gallstone formation. Alpha-thalassaemia, which is associated with reduced haemolysis, has also been shown to affect bilirubin levels. Another potential modulating factor is heme-oxygenase, a rate-limiting enzyme in the heme catabolic pathway that results in bilirubin production. While the severity of jaundice and cholelithiasis in SCD patients is predisposed by the inheritance of certain UGT1A genetic variants, inconsistencies have been observed. We investigated whether the modulating effects of HO1 and alpha-thalassaemia variants may explain these inconsistencies. Two hundred and sixty-three SCD patients attending specialist clinics in two hospitals were studied: King's 116 SS, 5 Sbetao, 59 SC; St Thomas 83 SS. Eighty-one ethnically matched subjects were recruited as controls (HbAA). Cholelithiasis data ascertained by liver ultrasound was available for a subset of patients (76 SS, four SC). Samples were genotyped for variants of UGT1A, HO1 and alpha-thalassaemia. Data was analysed according to the sum of (TA) repeats on both alleles for the UGT1A and HO1 genes. Regression analysis showed that serum bilirubin levels were strongly associated with UGT1A repeat length in all subjects ( $P < 0.0001$ ). Furthermore, the mean increase in serum bilirubin (21.3% for SS/Sbetao, 20.5% for SC) and cholelithiasis risk (86.5% for SS/Sbetao, 67.6% for SC) could be quantified per (TA) repeat. HO1 genotype did not affect serum bilirubin in patients or controls. Co-existing alpha-thalassaemia correlated negatively with serum bilirubin in SCD patients ( $P < 0.0001$ ) but not controls. This is the first time the relationship between UGT1A (TA) repeat length, serum bilirubin and cholelithiasis, and its moderation by co-existing alpha-thalassaemia has been quantified.

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### Levels of haptoglobin and haemopexin in patients with sickle and other haemolytic diseases, and haptoglobin genotype frequency

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Several studies have reported anhaploglobinaemia in sickle patients that disappears after crisis. They demonstrated low levels of haptoglobin in steady state, with low or undetectable haemopexin in crisis that recovered to a moderate level in steady state. Other studies of haptoglobin phenotype have suggested an increase prevalence of Hp1 in Sickle Cell disease.

Haptoglobin levels were determined for 24 patients with haemolytic disease, including 15 sickle patients, by measuring peroxidase activity in complex with haemoglobin at low pH using colorimetry. Haemopexin concentration was determined using radial immunodiffusion. Haemoglobin was measured using a Hemocue system. PCR haptoglobin genotyping was done for 46 sickle patients.

Haptoglobin was detectable in 7 of 24 patients (less than 0.2 g/l), including 3 of 15 Sickle patients (LOD less than 0.1 g/l). The normal plasma range is 0.5 to 2 g/l. The mean haemopexin level for Sickle patients was  $0.26 \text{ g/l} \pm 0.18 \text{ (SD)}$ , average maximum levels were 0.4 to 0.5 g/l. Normal plasma range is 0.7 to 0.8 g/l. Free haemoglobin levels did not always inversely correlate with haemopexin concentration in these patients. All of the four SC patients had haemopexin levels of greater than 0.44 g/l (Mean 0.56 g/l). SBo and SBpositive together had a mean of  $0.313 \text{ g/l} \pm 0.16 \text{ (SD)}$ . It was not possible to correlate haemopexin level to crisis recovery.

Numbers of haptoglobin genotypes in Sickle patients were 16, 24 and 6 for Hp1, Hp2/1 and Hp2 respectively. These frequencies are similar to those reported in African populations and do not suggest an increased frequency of a specific genotype.

We suggest haemopexin might be a useful marker for measuring success of therapeutic interventions in haemolytic disease as it is unaffected by *in vitro* haemolysis. Possible therapeutic interventions might include trying to increase Hp concentration to achieve benefits from its antioxidative and anti-inflammatory properties that may help to stabilise and prevent pain episodes.

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### Meeting standards for the clinical care of children and adults with sickle cell and thalassaemia disorders in Wales

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Wales is an area of low prevalence for the haemoglobin disorders with one city of high prevalence. The Cardiff and Vale Thalassaemia and Sickle Cell Anaemia Professional Advisory Group (PAG) met for the first time in January 2004. The remit was to improve, rationalise and consolidate the work of the core team, clarify each member's role and responsibility, identify gaps in quality standards and find



solutions as an informal network with colleagues around Wales. Barriers to service commissioning were the ill-defined nature of service need and the lack of acceptable benchmarks for quality control. By October 2006 clinical standards of care for adults and children with thalassaemia and for children with Sickle Cell Disorders had been published.

To discover the challenges faced by Health Care Professionals in meeting the standards. A meeting was arranged to discuss these with the authors of the guidelines and patient representatives. PAG also analysed hospital admission data undertook a questionnaire survey of Consultant Haematologists and Paediatricians regarding patient numbers and the level of service provided.

Attendance was multidisciplinary and involved Health Professionals from different parts of Wales. More than 60 patients had received regular treatment in the area over the last three years. The standards of care for these patients in Wales are uneven with little or no organisation outside South Wales. Significant numbers of Health Professionals reported challenges relating to the lack of a Paediatric Haematologist, the complexity of the disorders encountered, and the lack of adequate and appropriate genetic counselling, local guidelines and clinical support in managing these disorders.

Feedback endorsed the idea of an informal network with the delegates providing the initial framework. There is an urgent need for the implementation of the standards, with local centres and links to specialist centres, clinical networks, audit and monitoring in Wales.

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# **A pilot study to investigate the relationship between symmetric dimethylarginine and glomerular filtration rate in children with sickle cell disease**

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Sickle cell disease (SCD) is an inherited disorder of beta-globin synthesis of haemoglobin, resulting in a tendency for haemoglobin polymerisation and consequent vaso-occlusion, tissue hypoxia, and ensuing organ damage. The kidney is particularly sensitive to hypoxia and renal failure is a major cause of morbidity and mortality in SCD.

In SCD there is initial hyperfiltration followed by progressive decline in glomerular filtration rate (GFR). Routine estimation of GFR in children is primarily based on plasma creatinine measurements. Problems associated with plasma creatinine in SCD children include increase in concentration with body size, changes in tubular secretion, and the use of inappropriate normal ranges. Estimated GFR (eGFR),  $k \times \text{height (cm)}/\text{plasma creatinine } (\mu\text{mol/l})$ , where  $k$  is a constant dependent on the creatinine analytical method, compensates for changes in body size. However, the formula has never been validated in hyperfiltration or children with SCD. Recently, new GFR markers have been proposed, including symmetric dimethylarginine (SDMA), that may be independent of body size. In this pilot study we have tested the hypothesis that eGFR and/or SDMA allow reliable estimation of GFR in SCD.

Thirteen HbSS patients,  $x$  male, age range 10–20 years (mean age 15 years) attending the Evelina Children's Hospital were studied. The patients were on regular blood transfusion for stroke management and were referred to the paediatric nephrology service for renal investigation, including formal GFR measurement using plasma clearance of Inutest. The plasma samples were also used for the measurement of creatinine and SDMA by stable isotope dilution mass spectrometry. eGFR was calculated using  $k = 35$ .

Inutest GFR ranged from 70–175 ml/min/1.73 m<sup>2</sup>. There was a significant inverse correlation between SDMA and Inutest GFR ( $P < 0.01$ ). There was no significant correlation between either plasma creatinine or eGFR and Inutest GFR.

These early data suggest that SDMA might prove valuable in monitoring GFR in children with SCD.

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# **Prenatal diagnosis of Hb H hydrops fetalis due to compound heterozygosity for the Filipino alpha-0 thalassaemia deletion and Hb Adana, alpha 2 codon 59 GGC-GAC**

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Here we describe the first prenatal diagnosis by molecular genetic analysis for Hb H hydrops fetalis due to compound heterozygosity for the Filipino alpha 0-thalassaemia mutation (–FIL) and the highly unstable alpha 2-chain variant Hb Adana.

Until recently Hb H disease was thought of as a generally mild disorder. However it is now recognised that Hb H disease, particularly forms involving non-deletion mutations can result in a critical reduction in alpha globin chain synthesis, producing severe phenotypes requiring transfusions or even death.

The couple originated from the Philippines and were referred for genetic testing when routine antenatal haemoglobinopathy screening identified the mother as having haematological parameters consistent with alpha 0-thalassaemia trait and the father as having parameters consistent with alpha + thalassaemia trait. Gap-PCR showed that the mother was heterozygous for the Filipino alpha 0-thalassaemia deletion (–FIL) and alpha globin gene sequencing showed that the father was heterozygous for the alpha 2 codon 59 GGC-GAC mutation (Hb Adana). Only one case involving alpha 2 Hb Adana and the –FIL deletion has been described in the literature and this resulted in the phenotype of Hb H hydrops fetalis. Therefore the couple were offered prenatal diagnosis for two pregnancies, both unfortunately resulting in an affected fetus and subsequent termination.

The incidence of Hb Adana and other potentially severe non-deletional alpha + thalassaemia mutations is presently unknown in most populations but needs to be elucidated, so that their significance to public health can be evaluated.

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# **Mild haemolytic anaemia due to homozygosity for Hb Icaria, alpha 2 stop codon TAA-AAA**

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We present a case of homozygosity for the alpha 2 stop codon mutation TAA-AAA (Hb Icaria). Homozygous Hb Icaria is previously unreported.

The patient was a 51 year old female of Italian ancestry with a long history of being investigated for unexplained mild haemolytic anaemia. Her red cell indices were; Hb 95 g/l, RBC  $3.74 \times 10^{12}$ , MCV 88 fl, MCH 25.4 pg and her A2 and F levels were 1.7% and 1.8%

respectively. Initial investigations of her alpha thalassaemia status by Gap-PCR and Southern Blotting were normal indicating the absence of any of the common deletional forms of alpha thalassaemia. However recent further investigations by alpha-globin gene sequencing and MLPA revealed that she is homozygous for Hb Icaria. Like the other alpha 2 stop codon mutations (e.g. Hb Constant Spring and Hb Koya Dora), Hb Icaria is unstable and gives rise to the phenotype of alpha + thalassaemia trait.

Unlike Hb Icaria, the homozygous states for Hbs Constant Spring and Koya Dora have been described previously. Both give rise to a phenotype which is very unusual for homozygous alpha + thalassaemia. They are characterised by Hb levels from 90 to 110 The red cell count is relatively low with a mean of 3.9, the MCV tends to be normal (mean 88), while the MCH is only slightly reduced (mean 26pg). They also have mild haemolytic anaemia. Homozygous Hb Icaria seems to produce a similar atypical phenotype.

The reasons for the unusual phenotype associated with homozygosity for the alpha 2 stop codon mutations are incompletely understood. However the normal MCV may be related to over-hydration of the red cells due to abnormal volume control, which results from membrane damage caused by the oxidised abnormal alpha chains.

As homozygosity for an alpha 2 stop codon mutation does not produce a typical alpha thalassaemia phenotype it may not be considered as a possible cause of a patient's haemolytic anaemia, therefore cases of homozygous Hb Icaria (and the other stop codon mutations) may be under-reported.

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### **Avascular necrosis of the femoral head in HbSS children taking hydroxyurea**

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Avascular necrosis (AVN) of the hip is a recognised complication of sickle cell disease during childhood, and a significant cause of morbidity. The natural history and risk factors are better defined in adults, where advanced AVN is irreversible and may require hip replacement. In children, early stages of the condition may be reversible, particularly prior to fusion of the femoral epiphysis.

Hydroxyurea (OH-urea) has been used in this clinic since 1999 for children with frequent and severe vaso-occlusive crises. During this time period, thirty one children under the age of 16 have been treated (median age 12.1 years, 18 males). The median (IQ range) of treatment duration at censorship (treatment terminated or 31/12/06) was 3.3 years (2.1–4.4). Concurrently, between 1999 and the end of 2005, 227 children under 16 years of age with HbSS, born between 1983 and 1999 have been followed in this clinic (104 patient years with hydroxyurea, and 1485 patient years without hydroxyurea). There were three cases with symptomatic AVN of the hip (stage 3 or 4) in children not taking OH-urea (incidence 0.2 per 100 patient years follow-up) compared with the 5 cases while on OH-urea (incidence 4.8 per 100 pt years follow-up). AVN was diagnosed after a median of 2.7 years on therapy. The risk by Kaplan Meier analysis, after 5 years of therapy was 17.4%. In all cases, the femoral epiphyses had not fused at the time of diagnosis. OH-urea was stopped, and two have been started on regular transfusion therapy with symptomatic and radiological improvement.

These observations suggest that hydroxyurea therapy in children may increase the risk of AVN, perhaps by increasing blood viscosity

and reducing blood supply to the femoral head. Children on hydroxyurea should be observed carefully and considered for alternative therapy if this complication occurs.

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### **Extracranial carotid artery occlusion in children with sickle cell disease**

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Cerebral artery stenosis and occlusion is observed in 10–20 per cent of children with HbSS, and predisposes to ischaemic infarct. The lesions are usually seen in the intracranial portions of the internal carotid artery (ICA) as well as the proximal middle cerebral artery (MCA) and anterior cerebral artery (ACA), and are identifiable by transcranial doppler ultrasound (TCD) and magnetic resonance angiogram (MRA). We are aware of only two patients reported in the literature with stenotic lesions in the neck vessels, one case involving the common carotid, the other involving the vertebral artery. This paucity of reports may be due to the rarity of these lesions, or because recommended neuro-imaging in HbSS children does not routinely include the neck vessels.

We have now observed four such children, three presenting with acute infarctive stroke with internal carotid artery occlusion and one found incidentally on MRA, during investigation of increased TCD velocities. The diagnoses were made by magnetic resonance angiography of the neck and head. Their clinical features and radiographic findings will be presented.

The children with stroke were initially anti-coagulated, as it was not possible to exclude carotid dissection. All four are now on long-term transfusion. Follow-up scans have been done on two and both show persisting extracranial occlusion. There have been no further episodes of cerebral infarction in the affected children.

We suggest that internal carotid artery occlusion or dissection should be considered in children with sickle cell anaemia presenting with stroke, and they should have imaging of the neck vessels as well as cranial MRI and MRA. Consideration should be given to anti-coagulation in those with extracranial carotid occlusion. Furthermore, children with high velocities on TCD should also have doppler ultrasound of the neck vessels.

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### **Risk factors for stroke recurrence in sickle cell disease**

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**Introduction:** Regular blood transfusion to maintain haemoglobin S below 30% reduces the risk of recurrent ischaemic stroke in sickle cell disease (SCD) from 70% to 10%. Risk factors for recurrence

despite transfusion include absence of a prodromal illness before the first stroke and presence of occlusion or severe stenosis of the basal cerebral vessels with moyamoya collaterals, but it is not clear whether these are independent.

**Methods:** Medical records were retrieved for London patients with SCD with ischaemic stroke (acute neurological event with infarction on neuroimaging) between 1981 and 2001 and had been on a regular transfusion program to prevent recurrence. Survival analysis was used to examine the effects on recurrence risk of the presence of moyamoya on angiography (magnetic resonance or conventional) soon after the first stroke and a prodrome at the time of the first stroke.

**Results:** Of 96 patients (92 HbSS; 46 male) with a first stroke at a median age of 5.9 (range 1.1–19.7) years, 44 had had a prodrome (10 chest crisis, nine pain, eight fever, four parvovirus associated aplastic anaemia, four meningitis, two dehydration and one head injury) beginning 1–10 days previously. Twenty-one had moyamoya collateral on initial angiography. After median follow-up of 3.89 years (range 1 day to 20 years), forty-five had a further event (23 transient ischaemic attack, 22 stroke), and four died. In multivariable Cox regression, risk of recurrence was less for those with a prodromal illness before the first stroke (hazard ratio, [HR] 0.45, 95% confidence intervals [CI] 0.23, 0.89) and independently greater for those with moyamoya collateral (HR 2.12, 95% CI 1.14, 4.11).

**Discussion:** Moyamoya collateral on angiography and absence of a prodrome are independent risk factors for recurrence in children with SCD and stroke. In selected patients, revascularisation surgery might reduce the risk of further neurological events.

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### Auto-immune haemolytic anaemia and intracranial vasculopathy in Down syndrome

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Haemolysis appears to be a risk factor for pulmonary hypertension in haemolytic anaemias. Cerebral vasculopathy might also be associated.

A 4-year-old girl with Down syndrome presented with a right-sided hemiparesis and expressive dysphasia a few days after developing an upper respiratory tract infection (URTI). Aortic coarctation and an atrio-ventricular septal defect had been repaired in infancy. She had had idiopathic thrombocytopenic purpura (ITP) at the age of 2 and a history of mild asthma and recurrent URTIs associated with pallor but not jaundice.

Haemoglobin was 80 g/l with slightly elevated reticulocytes ( $112 \times 10^9$ , normal  $10\text{--}100 \times 10^9$ ). Lactate dehydrogenase (LDH) was elevated (962 IU/l, normal  $<500$  IU/l). Warm IgG and C3d antibodies were detected, haptoglobin was 0.0583 and DAT test was positive. The bone marrow was hypercellular and showed a left shift in granulopoiesis. A polyclonal B-cell population raised the suspicion of autoimmune lymphoproliferative syndrome but T-cell subsets were normal double and negative T cells were absent.

Echocardiography demonstrated elevated right ventricular pressures (55 mmHg). Transcranial Doppler showed right and left internal carotid/middle cerebral artery (ICA/MCA) velocities of 250 and 180 cm/s respectively (normal  $<140$  cm/s).

She was commenced on prednisolone but 2 weeks after weaning, she had a further episode of slurred speech and unsteadiness. Her TCD velocities remained high and she had further episodes of haemolysis treated with steroids at 8 and 10 months after initial presentation; during the second, she had a further stroke and

received a blood transfusion. Angiography confirmed occlusive vasculopathy and she underwent right-sided external-internal carotid artery revascularisation. Fifteen months later, she had no further episodes, and was making development progress along her previous trajectory, with Prednisolone cover for URTIs.

Stroke has been reported in several haemolytic anaemias. Once haptoglobin is saturated, nitric oxide is scavenged by oxyhaemoglobin, leading to vasoconstriction and eventually to irreversible vasculopathy and ischaemia.

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### Transcranial doppler in sickle cell disease in Africa

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**Background:** Although sickle cell anaemia (SCA) is one of the commonest genetic disorders in the world, there has been limited clinical research in Africa. Measuring cerebral blood flow velocity by Transcranial Doppler (TCD) is a sensitive and specific tool in stroke prediction.

**Methods:** TCD was performed in patients recruited from the outpatient clinic in Kilifi, Kenya.

**Results:** In 140 patients with SCA, ages 3 months to 16 years, the median time averaged mean velocity (Vmean) was 116 cm/s (SD 38, range 0–219 cm/s) compared with 97 (SD 24, range 46–190) cm/s in 142 controls aged two months to 14 years ( $P = 0.0001$ ). Twenty-eight SCA patients (20%) had Vmean greater than and 16 (11%) had Vmean less than two standard deviations from the mean for controls in one or both ICA/MCAs, but only seven (5%) had a velocity above 170 cm/s (one  $>200$  cm/s), with the highest proportion aged between 5–9 years ( $P = 0.02$ ). Forty-five (32%) SCA patients had a second TCD, two 14 years later and the remainder 2 years later. Of the 21 restudied who had high Vmean at baseline, 14 remained high and 2 became low. Of 15 restudied who had low Vmean at baseline, 14 remained low and none became high. Patients with abnormal velocity had lower oxygen saturation ( $P = 0.01$ ) and haematocrit ( $P = 0.05$ ). Although abnormal Vmean was also associated with lower haemoglobin level, red blood cell count and high white cell count, this was not statistically significant. Neurology included history of convulsions in 25 (14%) and of CVA in 4 (2%). There was a trend for a greater increase in Vmean ( $P = 0.06$ ) in three patients with convulsions in the interim.

**Conclusion:** A relatively small proportion of African SCA patients have conditional or abnormal velocities or stroke, although convulsions are common. Low velocities, perhaps secondary to moyamoya, are common.

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### Nocturnal autoCPAP for sleep-disordered breathing in sickle cell disease: pilot data

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Young people with sickle cell disease at high risk of sleep disordered breathing and there is some evidence for an effect on severity. However, surgery has long waiting lists and may not work. Conventional CPAP has proved difficult to use in children, but autoCPAP, which only triggers when the patient obstructs, might

have advantages. We report the use of and compliance with auto CPAP in a child with sickle cell anaemia over a one year period and the development of a protocol for a pilot study.

The patient is 13 and arrived in England at the age of 10. There were symptoms of upper airways obstruction, with loud snoring every night and mouth breathing. Overnight pulse oximetry showed a mean overnight oxyhaemoglobin saturation of 93% with frequent dips. A trial of autoCPAP was instituted with a clinical endpoint of an improvement in pain frequency. The patient hoped Mathematics scores might improve.

The patient tolerated seven nights a week initially but then asked to use the autoCPAP for schoolnights only, with compliance for one year despite some discomfort from the mask. Exercise tolerance,

concentration and daily pain improved and Mathematics improved by one grade.

Based on this experience, the pilot phase of a randomised controlled trial has commenced at Kings College hospital (PI Dr David Rees). The primary hypothesis is that nocturnal desaturation is associated with low Processing Speed Index and that this morbidity can be reduced with auto CPAP and/or overnight oxygen supplementation. Secondary endpoints are sleep quality, assessed with the Chervin sleep questionnaire, internal carotid/middle cerebral artery velocity measured using transcranial Doppler and attention using Connor Continuous Performance Test. This pilot phase will also explore feasibility, acceptability, compliance, dosage, safety, particularly with respect to the potential toxicity of oxygen, and the use of texting to document pain frequency.

## Poster Presentations: Transfusion

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### Fresh frozen plasma (FFP) from male donors – the impact on reported incidence of transfusion related acute lung injury (TRALI) in the UK reported to the Serious Hazards of Transfusion (SHOT) scheme CE Chapman\*, LM Williamson\*, H Jones\*, H Cohen\* and D Stainsby\*

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From 1996 to 2005, 185 cases of suspected TRALI were reported to SHOT; 38 fatal, 110 causing major morbidity. Case definition is 'acute dyspnoea with hypoxia and bilateral pulmonary infiltrates during or in the 24 hours after transfusion, with no other apparent cause'. All cases should be discussed with the Blood Service, to ensure that patients and donors are investigated for leucocyte antibodies and antigens, with the aim of identifying the implicated component and determining imputability.

There were 49 cases assessed as highly likely or probable TRALI where FFP or platelets were implicated, compared with 11 for implicated red cells; an incidence of 1:76 000 for FFP/platelet units and 1:1 440 000 for red cell units issued from UK Blood Services.

In 2003, the English National Blood Service, which provides >80% of UK blood supplies, implemented a policy of using plasma from male donors, as far as processing restrictions allow, to produce FFP and for suspension of buffy coat derived platelet pools. This has been achieved for >90% FFP units and >85% of platelet pools. Female plasma in stock was not withdrawn.

TRALI cases reported to SHOT since 1996 are analysed to assess the impact of this strategy. The number of reports of TRALI graded as possible or higher has fallen in 2004 and 2005, as have TRALI related deaths. This decrease is entirely due to a reduction in cases associated with FFP and platelets. Female donors still accounted for all cases in 2004 and 2005 where a significant matching antibody was found.

Year	96/ 97	97/ 98	98/ 99	99/ 00	00/ 01	*01/ 02	2003	2004	2005
<i>Number of reports</i>									
Total all causes		190	244	292	282	480	454	540	609
TRALI reports	9	14	16	18	13	33	36	23	23
meeting case definition									
Highly likely/probable	N/A	N/A	N/A	6	8	18	22	13	6
Possible	N/A	N/A	N/A	9	2	14	6	4	4
<i>Implicated components in highly likely/probable TRALI</i>									
FFP	N/A	N/a	N/a	3	4	8	8	6	1
Platelets	N/A	N/a	N/a	1	1	3	8	4	2
RBC	N/A	N/a	N/a	1	3	1	1	3	2
FFP + other	N/A	N/a	N/a	1	0	6	3	0	0
Cryoprecipitate	N/A	N/a	N/a	0	0	0	2	0	1
Deaths at least possibly due to TRALI:	2	2	4	4	3	7	7	3	3

\*15 months data due to change in reporting yearN/A not assessed

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### A retrospective study on transfusion practice in myelodysplasia and myelofibrosis: includes effect of age of blood and type (BAT v TT)

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Little is published on transfusion practice in myelodysplasia. This is a retrospective study at a single institution including 23 patients with myelodysplasia and examining 185 transfusion episodes. Results showed that the average transfusion 'trigger' Hb was 9.5 (7.2–11.8,95% CI). Patients with symptoms of anaemia had significantly lower Hb (9.1 vs 9.8,  $F = 5.3$ ,  $P = 0.002$ ). The age of blood transfused had no significant effect on the transfusion interval or change in HB. However most blood was >15 days old and younger blood tended to be used on patients with antibodies which may confound results. The type of blood transfused (BAT vs TT) had no measurable effect on transfusion interval or change in Hb. However the number of patients is small and this does not exclude an effect but does suggest any effect would not be clinically significant.

In conclusion increasing the transfusion trigger Hb for patients may alleviate symptoms. A further study is needed to ascertain if this

would also increase the number of transfusions or just involve more intensive transfusions initially to raise the Hb threshold.

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### Haemolytic disease of the fetus and newborn due to anti-Fy<sup>a</sup>: further evidence of severely affected pregnancies

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Maternal alloimmunisation against Duffy (Fy) red cell antigens, usually Fy<sup>a</sup>, has rarely been reported to cause severe haemolytic disease of the fetus and newborn (HDFN). Two recent cases of severe HDFN associated with anti-Fy<sup>a</sup> prompted us to review all cases monitored by the South-West regional Fetal Medicine Unit and Red Cell Reference Laboratory between 2000 and 2006.

Sixty-one pregnancies were identified. Forty-five of these had anti-Fy<sup>a</sup> titres rising above 32, the threshold for referral for specialist assessment and counseling. Two cases were referred for intra-uterine transfusions(IUT). The first case was blood group O R1R2 and in her fourth pregnancy. Anti-Fy<sup>a</sup> titre rose to 2000 at 20-weeks gestation and evidence of fetal anaemia was seen on scan at 28-weeks. The fetal haemoglobin prior to transfusion was 5.3 g/dl and rose to 16 g/dl after 60 mm of packed red cells. She had an emergency section at 32 weeks. The cord direct antiglobulin test was strongly positive and the baby required a top-up transfusion. The second case was maternal group O R1R1. She was in her sixth pregnancy having had a baby previously affected by HDFN due to anti-Fy<sup>a</sup> who required a top-up transfusion. Evidence of fetal anaemia and reduced fetal movements were seen at a 35-week scan with anti-Fy<sup>a</sup> titre 256. Fetal blood sampling showed the fetal haemoglobin to be 13.5 g/dl which is on the 10th centile for gestational age. The baby was delivered by caesarian section the following week, had a strongly positive direct antiglobulin test and required phototherapy.

These cases show that severe HDFN can be associated with anti-Fy<sup>a</sup> and our data suggest that 1% of affected cases may require IUT.

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### In vitro assessment of a novel pooled granulocyte component produced from whole blood donations: an improved component for clinical use

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The number of granulocyte concentrates transfused in England is increasing. Whole blood derived granulocytes (buffy coats) may be issued as an alternative to apheresis donations. Buffy coats are however not only heavily contaminated with red cells and platelets but there is also minimal data describing their functionality. We developed a method to produce a purer pooled granulocyte component (PGC) from whole blood donations and assessed its cell content, neutrophil viability and function. The PGC was prepared by pooling 400 ml of SSP + platelet additive solution and 10 ABO-matched buffy coats. Following centrifugation, the leucocyte-rich layer was removed, 160 ml ABO-matched plasma was added and the PGC irradiated (25–50 Gy). Neutrophil viability, chemotaxis, phagocytosis and oxidative killing activity were determined by flow cytometry. Results from 13 PGC 16–18 hours following donation were compared with those obtained from 20 standard individual buffy coats 12–18 hours following donation, and with fresh whole blood.

The PGC contains similar numbers of neutrophils with a reduced volume and haemoglobin content when compared to 10 individual buffy coats. Neutrophils in the PGC maintain >90% viability, oxidative burst and phagocytic activity and their ability to migrate towards a chemoattractant 16–18 hours following donation. This compares well with neutrophil function in fresh whole blood and standard buffy coats. Six PGC have been transfused to three patients without adverse clinical sequelae. Clinical studies assessing the safety and dose of the PGC are in progress.

	Fresh CPD whole blood (n = 18)	Buffy coat @ 12–18 hours (n = 20)	PGC @ 16–18 hours (n = 13)
Viability (%)	> 95	96.9 (88.3 to 99.2)	98.8 (97.7 to 99.2)
pH	–	7.08 (7.02 to 7.22)	7.06 (7.02 to 7.11)
Volume (ml)	–	58 (55 to 65)	250 (232 to 272)
Haemoglobin (g/unit)	–	7.93 (5.88 to 10.15)	15.25 (12.53 to 19.58)
Granulocyte con- tent ( × 10 <sup>10</sup> )	–	0.11 (0.043 to 0.20)	0.88 (0.70 to 1.17)
Phagocytosis (% PMN positive)	93.3 (82.5 to 98.6)	64.6 (82.1 to 97.0)	94.3 (86.0 to 98.7)
Oxidative burst (% PMN positive)	87.1 (73.2 to 98.9)	88.7 (77.4 to 95.2)	89.6 (35.2 to 96.1)
Chemotaxis (% PMN migrated)	Control 0.6 (0.4 to 0.9) fMLP 6.8 (1.7 to 17.7)	Control 0.08 (0.3 to 2.7) fMLP 4.1 (1.5 to 14.3)	Control 1.8 (0.5 to 4.2) fMLP 7.8 (1.9 to 14.4)

Date = Median (range)

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### The effect of the Sugarbaker procedure on haemostasis

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Pseudomyxoma peritonei is a progressive disease of the peritoneum, characterised by the gradual accumulation of mucinous fluid in the peritoneum. Left untreated it is ultimately fatal. Although no cure exists, the Sugarbaker procedure combining complete cytoreduction, intra-operative hyperthermic intra-peritoneal chemotherapy and post-operative intra-peritoneal chemotherapy, has shown promising results.

The aim of this study was to assess haemostatic function in this group of patients pre-operatively and the effect of the Sugarbaker procedure on haemostasis.

Citrated plasma was collected from 40 patients, pre- and post-operatively, and pre and 30 min post any blood components during surgery. Coagulation screening tests and whole blood global assay for haemostasis were performed. The prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (Fib) were performed on the MDA II (Trinity Bioscience). Thromboelastometry tests (Ex-TEM, In-TEM and Fib-TEM) were performed on the ROTEM (Biotis).

Pre-operatively patients had normal PT (mean = 13.5 s) and APTT (mean = 30.6 s) and raised Fib (mean = 4.7 g/l), suggesting the possibility of a hypercoagulable state.

The PT (mean pre-op = 13.5 s, post-op = 15.9 s), APTT (mean pre-op = 30.7 s post-op = 36.6 s), and fibrinogen (mean pre-op = 4.7 g/l post-op = 1.6 g/l) all displayed significant differences with *p* values of < 0.001. Thromboelastography demonstrated deterioration in clot quality as measured by the MCF (maximum clot firmness).

The PT, APTT and Fib pre and post FFP (fresh frozen plasma) showed no significant difference.

Results indicate that this procedure has a detrimental effect on haemostasis, identified by a reduction in fibrinogen and loss in clot quality as indicated by thromboelastography. This suggests that cryoprecipitate, rich in fibrinogen, may be a more appropriate blood component for use in association with this surgery.

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### **Use of the haemonetics OrthoPat<sup>®</sup> orthopaedic perioperative autotransfusion system reduces both blood usage and overall transfusion costs in patients undergoing revision hip arthroplasty (RHA)**

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In response to HSC2002/009 'Better Blood Transfusion' in May 2005 our institution introduced the use of the Haemonetics OrthoPat<sup>®</sup>

Orthopaedic Perioperative Autotransfusion System for patients undergoing RHA. There was an initial period of staff training however no additional theatre staff were required to operate the OrthoPat<sup>®</sup> system.

In the first quarter (May-July 2005) the OrthoPat<sup>®</sup> was only used in 12% (2/17) of cases resulting in total blood usage of 64 units and mean transfusion cost per case (including costs of the OrthoPat<sup>®</sup>) of £533. By the fourth quarter (Feb-April 2006) the OrthoPat<sup>®</sup> had been used in 50% (7/14) of cases with a total blood usage of 22 units and a mean transfusion cost per case (including the cost of the OrthoPat<sup>®</sup>) of £334. There was an overall mean reduction in blood usage of 58% and mean reduction in transfusion cost per case of 37%. The mean volume of blood salvaged was 234 mls (haematocrit 0.75–0.80) with a range of < 50 mls to 600 mls. Both the use of the OrthoPat<sup>®</sup> and the volumes of blood salvaged increased during the study period and the technology is currently being use in 75% of cases.

Our results show that the use of the OrthoPat<sup>®</sup> can reduce both the overall blood usage and mean transfusion costs in patients undergoing RHA. In our experience there was a gradual increase in the use of the OrthoPat<sup>®</sup> and a significant period of time following its introduction before we were able to demonstrate its potential benefits.

We also found the overall reduction in blood usage was greater than would have been expected from the volumes of blood salvaged alone suggesting the introduction of such technology may result in additional benefits such as a greater awareness of good blood transfusion practices within the orthopaedic directorate.

## **Poster Presentations: Transplantation**

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### **Risk factors, management and consequences of rhodotorula infections in immunocompromised haematology patients**

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Rhodotorula species are saprophytic yeasts belonging to the family Cryptococcaceae. They have been isolated from human skin, environmental sources and seem to have strong affinity for plastics. Rhodotorula species show low virulence but have been reported causing serious infections in immunocompromised patients, particularly in the presence of indwelling catheters.

Between August 2003 and September 2006, 12 episodes of rhodotorula fungaemia (defined as at least one positive blood culture) in 10 patients were documented within our Unit. All positive blood cultures were from Hickman lines. 50% of episodes were in patients who had been neutropenic for a prolonged time (10 days to 12 months), 58% in patients within 6 months of an allogeneic bone marrow transplant (all conditioned with MabCampath), 34% in patients who had undergone intensive chemotherapy before the infection, and 8% in patients who had received immunosuppressive medications during the 6 months before the infection.

In 11 of 12 episodes the line was removed when results of cultures were available. In one episode the line was not removed but infection recurred within 60 days requiring line removal. In 9 of 12 episodes, patients were started on intravenous antifungals. In the remaining 3

episodes, patients were kept on oral azoles. All patients survived the septic episode without major clinical consequences.

Most Rhodotorula infections are of low virulence. Presence of an indwelling catheter and immunosuppression are the main risk factors. Treatment with MabCampath appeared to be a possible risk factor. In 50% of the episodes, patients had a normal neutrophil count but they were significantly immunocompromised either because of concomitant treatment or underlying disease. Line removal is probably the most valuable therapeutic intervention and is sometimes sufficient to treat the septic episodes but, in some instances, the introduction of intravenous antifungals may be needed on the basis of the clinical picture.

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### **Echocardiographic findings in long term survivors of allogeneic stem cell transplantation for chronic myeloid leukemia**

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Long term cardiac complications following allogeneic stem cell transplantation include cardiomyopathy, impaired left ventricular function, cardiac dysrhythmias, valvular dysfunction and pericardial disease. Cardiac dysfunction may result from pre-transplantation toxicity due to induction chemotherapy (e.g. alkylating agents,

anthracyclines) the transplant conditioning regimen (chemotherapy and/or radiotherapy) or co-morbidities.

We have evaluated transthoracic echocardiography data from 22 patients (12 male) who had received myeloablative allografts for CML more than 10 years previously. All patients received cyclophosphamide and total body irradiation (TBI) as their conditioning regimen. Two patients received a second allograft procedure with chemotherapy only conditioning. The median age at the time of assessment was 48.5 years (range 34–70) and the median transplant to assessment time was 14 years (range 12–26 years).

The median fractional shortening in this group of patients was 35% (normal 29–37%) with a range of 26–42%. Four patients had mild impairment of their fractional shortening. One of these patients also had moderate mitral regurgitation and a history of hypertension. Three patients with normal fractional shortening were noted to have mild diastolic dysfunction.

Among the patients transplanted for CML described in this study, the prevalence of observed echocardiographic abnormalities was low and contrasts with patients transplanted for acute leukemia where a significant reduction in fractional shortening has been reported (Liesner *et al*, [1994] *Journal of Clinical Oncology*, 12, 916–24). This difference may be a reflection of the more intensive pre-transplant chemotherapy given to patients with acute leukaemia.

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# **Confirmation that ideal body weight is a safe method for calculating CD34 + stem cell dose for PBSC autograft in haematological malignancy**

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CD34 + dose is an important factor in determining rate of engraftment following stem cell reinfusion in PBSC autograft. Recent reports have suggested that there is a closer correlation between CD34 + cells/kg and haematopoietic reconstitution when the stem cell dose is calculated using ideal body weight (IBW) rather than actual body weight (ABW). We confirm these findings in 218 consecutive patients receiving PBSC autograft for haematological malignancy at a single centre. The median ABW was 74 kg compared to 62 kg when IBW was calculated, a 21% difference. Thus the recorded median CD34 + dose of  $5.0 \times 10^6/\text{kg}$  (ABW) rose to  $6.1 \times 10^6/\text{kg}$  when calculated by IBW. Neutrophils reached  $0.5 \times 10^9/\text{l}$  in 8–21 days (median 11) while platelets reached  $20 \times 10^9/\text{l}$  unsupported in 7–38 days (median 12). For both neutrophil and platelet engraftment a greater inverse correlation was seen when IBW rather than ABW was used to calculate stem cell dose ( $r^2 = 0.104$  vs  $r^2 = 0.082$  for neutrophils and  $r^2 = 0.135$  vs  $r^2 = 0.085$  for platelets). Those patients who did not achieve a CD34 + dose of  $4 \times 10^6$  cells/kg by ABW but did by IBW were shown to achieve almost equivalent neutrophil and platelet engraftment ( $z = -0.1$   $P = 0.018$ ) as those who achieved the target stem cell dose by both methods. In those patients treated for myeloma this finding was not confirmed due to the difficulty in accurately calculating IBW in patients who have lost skeletal height. Our study confirms that calculation of CD34 + cell dose by IBW safely predicts engraftment in PBSC autograft but we would suggest that pre-morbid height be used in its calculation in patients with myeloma. The major benefit of this method of calculation is that it reduces the absolute stem cell dose required for approximately 80% of patients and therefore allows some to avoid additional apheresis procedures or even courses of conditioning chemotherapy.

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# **The first case of optic neuritis preceding progressive outer retinal necrosis (PORN) after bone marrow transplantation (BMT): a severe manifestation of varicella-zoster virus (VZV) infection**

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Severe VZV infection causes PORN, resulting in devastating unilateral and bilateral visual loss, and occurs with immune suppression. It was first described in HIV patients, where regression is commoner. However, only three reported cases in BMT exist, where presentation, treatment and prognosis vary, being more severe, with refractory disease outside the orbit, causing permanent disability.

CASE: An 18-year-old woman with Philadelphia chromosome positive acute lymphoblastic leukaemia underwent allogeneic stem cell transplantation from an haplo-identical donor. Five months post-engraftment, VZV was isolated from left groin shingles, which resolved with intravenous aciclovir, and then valaciclovir. Three months later, the patient was admitted with two weeks of headaches and blurred vision in the right eye, with central visual loss over four days. Examination revealed no light perception, relative afferent papillary defect, Snellen 6/6 vision in the left eye and normal fundoscopy. Optic neuritis was diagnosed and treated with three days of intravenous methylprednisolone and oral prednisolone. Five days after treatment, blurred vision in the left eye developed. Repeat fundoscopic and slit-lamp appearances were initially confused for leukaemic infiltration. However, subsequent appearances were consistent with PORN. Orbital MRI confirmed optic neuritis and VZV DNA was detected by PCR from the CSF and vitreous humour.

Visual deterioration continued despite the combination of ganciclovir and foscarnet. Intravenous cidofovir and intra-vitreous foscarnet were added without success. Cerebellar signs and oscillopsia developed after four weeks, with disease extension. Other late complications included recurrent retinal detachment requiring multiple surgical interventions. The patient is now registered blind, reading Braille and studying literature.

This is the first case of optic neuritis preceding PORN in BMT, where treatment is particularly challenging. Optimal therapy is undefined. Distinction from acute retinal necrosis, CMV retinitis, and leukaemic retinal infiltration may prove difficult without vitreous and CSF VZV PCR.

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# **A standardized potency assay for stem cell products prior to transplantation**

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The potency or quality of a stem cell product for clinical transplantation is usually determined by total nucleated cell counts (TNC), viability, CD34 cell numbers and growth in the colony-forming assay (CFA). Results from the latter are subjective, cannot be standardized and require 14 days to be obtained. In contrast, an instrument-based, ATP proliferation assay (HALO) can produce

results in 5–7 days and is fully standardized so that intra- and inter-laboratory results can be directly compared. As lympho-haemopoietic cells proliferate in response to growth factors, the intracellular ATP (iATP) increases proportionately. After incubation in 96-well plates, the iATP is released and acts as a limiting substrate for a luciferin/luciferase reaction producing bioluminescence that is measured in a plate luminometer. In this study, two groups (HG and PSBC) performed TNC, viability, CFA and HALO on similar aliquots from 12 erythrocyte-depleted cord blood samples on the same day. CD34 was performed only at PSBC. The results were the following. A correlation existed for TNC between sites ( $r = 0.98$ ,  $P < 0.001$ ). A correlation ( $r = 0.79$ ,  $P = 0.02$ ) also existed between sites

for the CFA despite using different methodologies, but a good correlation between CFA and CD34<sup>+</sup> cells was only found at PSBC ( $r = 0.87$ ,  $P < 0.001$ ). A significant correlation ( $r = 0.82$ ,  $P < 0.001$ ) was observed at HG between CFA and HALO. The lack of correlation observed for the CFA with CD34 and HALO was due to differences in colony counting practices, thereby illustrating the how subjectivity can lead to inconsistent results. In contrast, when the results from HALO were compared, a significant correlation ( $r = 0.94$ ,  $P < 0.001$ ) was found between both sites. Despite the small number of samples, the results clearly demonstrate that measurement of proliferation potential using HALO is an alternative to measuring differentiation potential using the CFA technique.



**WEDNESDAY 2 MAY 2007**

## Free Communications: General Haematology and Molecular and Cellular Biology

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### A novel application of digital single nucleotide polymorphism (dSNP) technology to the identification of chromosome 13q deletions in multiple myeloma and chronic lymphocytic leukaemia

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Deletions of 13q14 are common in Chronic Lymphocytic Leukaemia (CLL) and Multiple Myeloma (MM). Such deletions are commonly identified using interphase Fluorescence *In Situ* Hybridization (FISH), but this technique may have limited application when applied to archival tissue or samples with heterogeneous cell populations such as MM. Consequently, there remains a need to develop a sensitive technique for accurate detection of 13q14 deletions in both fresh and archival tissue.

We report here the novel application of digital Single Nucleotide Polymorphism (dSNP) technology to detect and characterise 13q deletions in MM and CLL. We tested paraffin-embedded bone marrow biopsies and whole blood samples from 12 MM and 15 CLL patients respectively. We analysed heterozygous SNPs across the 13q14.2 to 13q32 region for the presence of skewed allelic ratios and tested results for statistical significance by sequential probability ratio analysis. Where possible, deletion analysis was also performed by FISH.

We identified 13q14 deletions in 6/12 (50%) MM and 12/14 (86%) CLL cases, demonstrating the utility of dSNP technology. FISH and dSNP data proved concordant in 4/6 MM and 10/14 CLL cases. With most discrepancies (5/6), 13q14 deletions detected by dSNP analysis may not have extended to the region covered by the FISH probes.

dSNP analysis in MM allowed detection of 13q deletions in archival tissue with minimal neoplastic cell infiltration without the need for prior plasma cell enrichment. Deletion analysis in this cohort indicated that the minimal deleted region (MDR) may localise to a 331,000bp region of chromosome 13q14.3.

Our results indicate that dSNP is a useful technique that can be applied to both fresh and archival tissues and has particular utility where numbers of neoplastic cells are limited. Using this approach, it may be possible to narrow the MDR for MM and CLL, potentially enabling identification of candidate tumour suppressor genes.

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### The telomerase-associated protein NOP10 is mutated in autosomal recessive dyskeratosis congenita

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Dyskeratosis congenita (DC) is a bone marrow failure syndrome associated with multiple somatic abnormalities and an increased risk of malignancy. Identification of the genes involved in of this disease

suggests that it may arise through a defect in the maintenance of telomeres. To date, mutations have been identified in the RNA component and the reverse transcriptase component of telomerase in autosomal dominant DC, and in the protein dyskerin in X-linked DC, which is a core component of H/ACA small nucleolar RNPs as well as the telomerase complex. Through homozygosity mapping in consanguineous families we have previously shown that the autosomal recessive form of the disease is genetically heterogeneous. In one large consanguineous family, we have mapped the disease locus to chromosome 15q14. The gene encoding NOP10, a small core component of H/ACA small nucleolar RNPs and the telomerase complex, maps to this region and was therefore an ideal candidate. We find that three affected individuals in this family, who present with classical mucocutaneous features of DC, are homozygous for a mutation causing an R34W substitution in NOP10; consistent with autosomal recessive inheritance, their asymptomatic parents are heterozygous for this mutation. This amino acid is highly conserved and lies at the site of protein-protein and protein-RNA interaction in the RNP complex. We show that affected individuals have very short telomeres and significantly reduced levels of TERC in peripheral blood, while heterozygotes for this NOP10 mutation (parents and three normal sibs) have intermediate telomere lengths and TERC levels. Using small interference RNAs, we demonstrate that a reduction of NOP10 transcript levels brings about a subsequent reduction in the amount telomerase RNA. In conclusion, these findings suggest that this unusual family represents the first description of autosomal recessive DC, due to mutation in another component of the snoRNP and telomerase complex.

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### Utility of early screening for JAK2 V617F mutation in patients with erythrocytosis or thrombocytosis in a District General Hospital

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We retrospectively analysed the diagnostic utility of testing for the V617F JAK2 mutation in 322 consecutive patients referred to the Royal Bournemouth Hospital, for either raised red cell indices ( $n = 247$ ) or raised platelet count ( $n = 65$ ), over a 43-month period during which red cell mass measurement was not routinely available.

Based on a preliminary assessment consisting of history and physical examination, full blood count, ESR, blood film, Pao2, abdominal ultrasound (159 cases) serum Epo (126 cases) and bone marrow biopsy (58 cases), polycythaemia was considered to be transient (71 cases), secondary (89 cases), P Vera (19 cases) or to have no apparent cause (68 cases). Essential thrombocythaemia (ET) was diagnosed in 39/64 patients with persistent thrombocytosis.

Most patients with secondary polycythaemia had a history of heavy smoking and/or alcohol consumption. Only two abdominal ultrasound scans yielded a clinically significant diagnosis (hypernephroma). Low serum erythropoietin levels (17/126) were found in all four groups of polycythaemic patients whilst high levels (4/126) occurred only in non-PV group. Bone marrow histology consistent

with PV and ET were observed in 13/13 and 10/28 respectively. All ET patients had a normal ESR.

V617F JAK2 mutation was detected in DNA extracted from leucocytes using a tetra primer ARMS PCR assay and quantitated by pyrosequencing. Results are available for 136 patients with erythrocytosis and 44 patients with thrombocytosis and showed presence of mutation in 16/17 with suspected PV, 0/34 with secondary polycythaemia, and 24/39 with suspected ET. A low-level mutation was seen in 5 polycythaemic patients not initially suspected of having a myeloproliferative disorder.

In summary our data would support testing for the JAK2 V617F mutation in patients suspected of having PV and ET based on clinical criteria, blood count and blood film. Recent data suggest that JAK2 negative PV patients and those with a low Epo level should be screened for exon 12 JAK2 mutations.

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### HOX genes are a major target for epigenetic mis-regulation in adult and childhood leukaemia

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Altered expression of key transcription factors plays a pivotal role in the development of leukaemia. To begin to assess epigenetic mechanisms responsible for such aberrant regulation we have analysed the role of DNA methylation in altered expression of members of the HOX gene family, which are key regulators of embryonic development and differentiation and have been implicated in normal and malignant haematopoiesis. In both adult myeloid and lymphoid leukaemia two members of the HOXA cluster (HOXA4 and A5) were found to be frequently inactivated by promoter hypermethylation (between 26–64% of cases). A further 12 HOXA, B and C cluster genes were, in contrast, found to be essentially devoid of increased methylation (except HOXA6 in CLL, where 34% of samples exhibited hypermethylation). HOXA4 and A5 were also frequently inactivated in childhood ALL and AML (39–79% of samples). However, in contrast to the adult leukaemias, all but one of the additional HOX genes analysed were also found to be common targets for hypermethylation in both ALL and AML (4–26%), suggesting that HOX genes are differentially regulated in childhood vs adult leukaemia.

Interestingly HOXA4 hypermethylation exhibits frequent correlations with poor prognosis in patients. Hypermethylation of HOXA4 correlates with progression to blast crisis ( $P = 0.007$ ) and poor response to imatinib in CML ( $P = 0.04$ ), with cytogenetic status in AML (38%, 78% ( $P = 0.002$  vs good), 100% ( $P = 0.0002$  vs good,  $P = 0.04$  vs intermediate) samples hypermethylated in good, intermediate and poor prognostic subtypes respectively) and correlates with IgVh mutational status ( $P = 0.003$ ) and poor survival in CLL (median survival 159 vs 199 months in hypermethylated and non hypermethylated patients, respectively). Furthermore re-expression of HOXA4 in a CML blast crisis cell line results in re-expression

of markers of myeloid differentiation. These results suggest that aberrant epigenetic regulation of HOXA4 may play a key role in the development of multiple types of leukaemia.

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### The novel protein JUNE-1 binds histone H3

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Erythropoietin (EPO) is the principal regulator of terminal erythroid differentiation. Recently expression of EPO and its receptor has been demonstrated in non-erythroid cells with a potentially controversial role in tumour development. To increase understanding of EPO downstream signalling events, differential display PCR was used to isolate EPO responsive genes in a murine model of terminal erythroid differentiation. June-1, a novel gene upregulated in response to EPO, was identified. JUNE-1 has significant identity with only one other human protein SPOC1/PHF13, also of unknown function but which demonstrates an association with residual disease and survival in ovarian cancer. The gene is widely expressed suggesting a role beyond erythropoiesis and although highly conserved across vertebrate species the function of JUNE-1 remains to be elucidated. A splice variant, missing a 201 bp section of exon 4, has also been identified. In silico sequence analysis reveals a nuclear localization signal (NLS) and a PHD motif (plant homeodomain). PHD domains act as zinc fingers and many PHD containing proteins have a role in chromatin regulation. Cellular localisation studies using GFP tagged JUNE-1 indicates that the protein is located almost exclusively in the nucleus. The splice variant which retains both the PHD domain and the NLS is also nuclear. Western blot analysis of immunoprecipitated JUNE-1 from transient over-expression in 293T cells reveals both tyrosine and serine phosphorylation suggesting additional regulation at the post-translational level. Some PHD fingers recognise modified histone residues which promote activation and/or repression of gene expression. JUNE-1 demonstrates an association with histone H3 suggesting it may function as a novel effector protein involved in regulating transcription.

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### Systems biology analysis of the G0-G1 cell cycle transition of human primary T cells

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We identified a Go-G1 commitment point in primary human T cells that controls entry into the cell cycle from quiescence. We demonstrated proof of principle that cellular pathways coupled during CD3/CD28 stimulation can be uncoupled experimentally. We have now used systems biology approaches to identify nuclear protein networks in primary human T cells that are regulated during the transition from quiescence into the cell cycle (Go-G1-S-phase).

First we sequenced chromatin and nuclear matrix proteins that became bound in G1 but were not bound in Go and vice versa by mass spectrometry. Bioinformatic analysis identified 76 proteins specifically bound in Go not G1 and 254 bound in G1 not Go. 179 of the 254 proteins bound in G1 not Go (i.e. dynamic protein changes)

were mapped to the 55,000 human protein interaction dataset. These are involved in numerous cellular functions, including epigenetics, transcription, RNA splicing and transport, and others. Cell cycle regulated chromatin/matrix binding of a subset was verified by western blotting (2/2 bound in G<sub>0</sub> not G<sub>1</sub> and 22/23 bound in G<sub>1</sub> not G<sub>0</sub>).

One of the proteins induced and bound in G<sub>1</sub> was SAP145 (SF3B2). This is a component of the ubiquitous SF3b RNA splicing complex, involved in both major (U2-type) and minor (U12-type) spliceo-

somes. Since SAP145 is induced during G<sub>1</sub> we investigated whether there was a role for SAP145 in regulating cell cycle progression. T cells depleted of SAP145 by siRNA enter G<sub>1</sub> from G<sub>0</sub> but progress poorly through S phase and die, probably by apoptosis. Proteins normally induced during G<sub>1</sub>, e.g. cyclin D3, Cdc6 and cdc2 are produced, suggesting that their pre-mRNAs are spliced normally. In contrast, the expression of p107 and cyclin A were reduced. Therefore, a systems biology approach to analysing cell cycle transitions identifies SAP145 as rate-limiting for the G<sub>1</sub>-S phase cell cycle transition.

## Free Communications: Paediatric Haematology 2

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### Management of over-anticoagulation in children

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Recent BCSH guidelines have addressed the management of adults over-anticoagulated with warfarin. However, despite increasing numbers of children taking long term warfarin there is a lack of data on the incidence and management of over-anticoagulation in this population. We have carried out a retrospective review of anticoagulation practices in our hospital and describe the management of children with INRs >5.0.

Data was collected retrospectively for 86 children on long-term warfarin between September 2001- September 2006. 53 children had undergone a Fontan's procedure and 19 had prosthetic heart valves.

Two hundred and thirty-six of one thousand and ten INRs done in the five year period (2.3%) were >5.0 (INR 5-8: 211, 8-10: 12, >10: 13). These episodes were managed by: a) warfarin dose reduction ( $n = 98$ ), b) dose omission ( $n = 116$ ), c) oral Vitamin K ( $n = 14$ ) and d) IV Vitamin K ( $n = 8$ ). Mean INR values for these 4 groups were 5.5, 6.3, 9.5 and 9.4. Therapeutic INRs were achieved within 24 hours in 39%, 33%, 20% and 25% respectively. Six per cent of INRs were sub-therapeutic following dose reduction or omission, 75% following IV Vitamin K and 50% following oral Vitamin K. Vitamin K was usually one tenth of the dose of warfarin previously administered though the range varied between 0.1 and 3.0 mg.

Minor bleeding complications occurred in 3 patients only.

High INRs occur infrequently in children and most can be safely treated in the absence of bleeding by a reduction or omission of the dose of warfarin. We have noted variability in management of high INRs, with less experienced clinicians more inclined to stop warfarin or administer vitamin K. This has led to subtherapeutic INRs which can be hazardous for children with prosthetic heart valves. As a result of this study, a more standardised approach to the management of over coagulated children has been introduced in our hospital.

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### Use of alternate stem cell sources for allografting in malignant infantile osteopetrosis: single centre experience

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Malignant infantile osteopetrosis (MIOP) is a lethal disorder of osteoclast dysfunction. Haematopoietic stem transplantation is the

only curative treatment currently available. The best results are seen with matched sibling donors; however the use of alternate donors is being explored. Early allografting is essential in order to minimise permanent neurosensory deficit. We studied 5 children with MIOP who underwent 8 allograft procedures in our unit in 3 years. The mean age was 18 months (2-53 months). Donors were matched sibling ( $n = 1$ ), unrelated cord ( $n = 2$ ) and haploidentical parent ( $n = 5$ ). The mean CD34 dose was  $8.4 \times 10^6/\text{kg}$  and a dose of  $10 \times 10^6/\text{kg}$  was achieved with all haploidentical grafts. Haploidentical grafts were T cell depleted and patients received serotherapy (ALG in four and OKT3 in one). Common conditioning regimes were used. Cyclosporine was used for graft-versus-host disease (GvHD) prophylaxis. Neutrophil and platelet engraftment was seen at a mean of 15 days (range 11-20 days) and 49 days (range 12-60 days) respectively. Three episodes of primary graft rejection occurred in two patients who then engrafted successfully after a haploidentical graft. Three patients showed complete chimerism (CC) at day 28 and this has persisted. Two patients that showed increasing mixed chimerism (i-MC) were treated with escalating donor lymphocyte infusions (DLI) with fludarabine resulting in CC in one patient and stable mixed chimerism in the other. Four patients developed grade two acute skin GvHD (2 post-DLI), one patient developed chronic skin GvHD. Clinical improvement or stabilisation was seen in four patients. Disease progression occurred in one patient in spite of CC. There was no transplant related mortality. One patient developed acute immune thrombocytopenic purpura needing immunoglobulin therapy. Hypercalcemia needing pamidronate was seen in one patient. Both have resolved. We have shown that the use of alternate stem cell donors, especially haploidentical parents, in the absence of a fully matched sibling donor, is a safe, readily available and effective transplant option that is likely to improve the outcome in children with malignant osteopetrosis.

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### Evaluation of analgesia regime with intranasal diamorphine and oral morphine for acute painful sickle crises in children

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Pain control for children with SCD is often inadequate and delayed. Use of injected opiates is frequent but uncomfortable, and may lead to increasing reliance on large injected doses later in life. We have designed a protocol for immediate and sustained analgesia avoiding injected opiates, relying on rapid assessment and treatment by the paediatric casualty nurse.

On arrival, intranasal diamorphine (0.1 mg/kg) is given with oramorph (0.4 mg/kg). Oramorph is repeated at 1,3,6 hours and 3 hourly prn thereafter. MST 1 mg/kg bd is added at 6 hours if pain is inadequately controlled. Treatment failures are given iv PCA morphine. We have evaluated the protocol by auditing our admissions between September 2004 and August 2005. We have also undertaken a questionnaire survey to assess subjective perception by patients/parents.

There were 96 admissions in 38 children. 13 did not require morphine. Average age was 11 years (4–16). There were 23 males and 15 females, 36 with HbSS and 2 HbSC. Time to intranasal diamorphine was: immediate in 44 (53%), and within 10 min in 21 (25%). There were deviations of the protocol in 24 admissions. Average dose of oramorph was 127.36 mgs (6–946 mgs). MST was required in 25 episodes (29%). Average dose was 267.4 mgs (40–760 mgs). On eight admissions in four patients PCA was required. In 72 evaluable episodes, median time to pain relief (mild or no pain) was 20 hours (IQ range 12–70). Median duration of admission was 3 days (IQ range 2–6). The regime was well tolerated, side effects included constipation, 15 (17.4%); vomiting, 7 (8%); pruritus, 13 (15%); and drowsiness: 1 (1.2%). Four episodes were complicated by chest crises.

In conclusion, this is an effective and practical protocol, which circumvents the need for injected analgesia. It could be compared with standard use of parenteral morphine in a randomized controlled study.

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### Acute chest syndrome in children with sickle cell disease: an inner London experience

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Acute Chest Syndrome (ACS) is diagnosed in a child with Sickle Cell Disease (SCD) when a new infiltrate on chest radiograph is accompanied by acute respiratory symptoms. It is the leading cause of death and the second most common cause of hospitalisation in children with SCD. ACS accounts for 20–30% of all sickle cell admissions in children and up to 15% of those are reported to require intensive management and mechanical ventilation. Most reports come from large, multi-centre studies from the USA and there is limited data on the pattern of illness and its management in the UK.

We set out to describe the pattern of ACS in children by reviewing retrospective data from a Central London teaching hospital with over 400 children with SCD who account for 110 acute admissions per year. A total of 29 episodes of ACS in 24 children were identified, four with pre-existing asthma and three with severe gastro-oesophageal reflux disease.

The age range was one to 15 years (median five years); 20 (69%) were female. Length of stay was from one to 27 days (median four days). Eighteen (62%) were admitted with a diagnosis of ACS. The remaining cases were initially admitted for acute painful crises or surgery, but developed acute chest syndrome while in hospital.

The most common clinical features were fever (median 39 degC) and low oxygen saturation (median 92%). Eight cases (28%) received blood transfusion. Only three (10%) patients were transferred to the Intensive Care Unit for further management. There were no reported deaths.

ACS is a significant cause of hospital admission and morbidity in children with SCD, and a third of cases developed ACS after being admitted for a different condition. A less severe pattern is observed than has been reported previously.

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### The Shwachman–Diamond protein is required for translational activation of ribosomes

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Shwachman–Diamond syndrome (SDS) is an inherited bone marrow failure disorder associated with pancreatic exocrine insufficiency, metaphyseal chondrodysplasia and leukaemia predisposition. As the cumulative risk of MDS/leukaemia is around 36% by 30 years, SDS represents an important paradigm for understanding the genetic determinants underlying the multi-step progression to leukaemia. The majority of SDS patients carry mutations in the *SBD* gene that encodes a highly conserved 250 amino acid protein of unknown function. Indirect evidence suggested that SBD might function in ribosome biogenesis. In particular, X-ray crystallographic studies revealed structural homology between an archaeal SBD orthologue and two yeast ribosome-associated proteins, Yhr087w (which co-precipitates with 60S ribosome biogenesis factors), and elongation factor 2 (the eukaryotic homologue of the bacterial GTPase elongation factor G). By taking advantage of the powerful genetic tools afforded by the model organism *Saccharomyces cerevisiae*, we have elucidated the conserved function of the yeast SBD orthologue Sdo1. We demonstrate that Sdo1 is critical for the release and recycling of the nucleolar shuttling factor Tif6 from cytoplasmic pre-60S ribosomes. This is the final step in the maturation of 60S subunits and is required for the translational activation of ribosomes. Through the application of genome-wide synthetic genetic array mapping technology, we identified multiple *TIF6* gain-of-function alleles that suppressed pre-60S nuclear export defects and cytoplasmic mislocalisation of Tif6 in SDO1 null cells. We show that Sdo1 functions together with the GTPase elongation factor-like 1, in a pathway that controls the translational activation of ribosomes. Our data link defective late 60S ribosomal subunit maturation to an inherited bone marrow failure syndrome associated with leukaemia predisposition. As ribosome dysfunction has also been described in Diamond–Blackfan anaemia, dyskeratosis congenita and cartilage hair hypoplasia, our data suggest that common molecular mechanisms may underlie the pathogenesis of these inherited bone marrow failure syndromes.

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### UK validation and initial testing of an ITP quality of life instrument

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Children with ITP need to adapt their lifestyle to minimise bleeds whilst awaiting spontaneous remission or curative therapy. Little is

known about how ITP affects the quality of life (QoL) of the child or family. Successful treatment may improve QoL or conversely may worsen QoL if excessive side effects develop.

We describe the 'translation' of an ITP-specific QoL tool (KIT), developed in Canada, into use for children from the UK. The KIT consists of 26 questions. A version is completed by children of seven years or older. Parents complete a proxy version and a parent impact KIT. The KIT produces scores with a range of 0 (worst) to 100 (best).

We have tested the KIT on 10 children with ITP. Prior to the final version all the children and 86% of the parents reported misunderstandings with the original version. Eight changes were

made from the original Child/Proxy KIT and five changes in the Parent Impact.

Worrying about the ITP getting worse, concerns about appearance, constitutional symptoms and changes in mood were highlighted as particular problems. Children with a platelet count below 20 had a significantly worse QoL than children over 20 (67 vs 85,  $P < 0.05$ ).

Cross-cultural testing of a North American measure in the UK resulted in important changes to the measure. We plan to incorporate the KIT into the UK registry [www.uk-itp.org](http://www.uk-itp.org). Ultimately the KIT will be an essential outcome measure in clinical trials and may aid in making decisions on whether or not to treat.

## Free Communications: Haemostasis and Thrombosis

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### **Incidence of bleeding and thrombotic events and pregnancy complications in cohort of families with dysfibrinogenemia secondary to Arg-16-His mutation** EK Nicholson\*, M Hills†, G Dolan† and RC Tait\*

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The dysfibrinogenemias are a group of inherited disorders characterized by fibrinogen molecules that are abnormal in structure and function. As a group these disorders exhibit heterogeneous clinical behaviour. The most common reported mutation is the substitution of histidine for an arginyl residue at position 16 (Arg-16-His) which is associated with a mild clinical phenotype. Published data suggests that 25% of affected patients have haemorrhagic symptoms, 5% have thrombosis (with known risk factors) and 3% have thrombosis without other known risk factors. Rates of spontaneous abortion and postpartum haemorrhage are also increased. However the apparent clinical significance may be overestimated due to case finding bias. We have identified 17 families with the Arg-16-His mutation, many incidentally through routine pre-surgical coagulation screening. We undertook a comparison of incidence of thrombotic and haemorrhagic events and pregnancy complications in these individuals and unaffected relatives.

Thirty-five individuals (27 affected; 8 unaffected) were assessed from the 17 families. Bleeding scores [Tosetto et al, *Journal of Thrombosis and Haemostasis* 2006; 4:766-733] in affected individuals ranged from -2 to 18 (mean 5, median 3) and in unaffected individuals from -3 to 4 (mean 1, median 0). An abnormal bleeding score ( $>3$ ) was more common (55% vs 12.5%) in the affected individuals ( $P = 0.05$ ). Venous thromboembolism occurred in 11% of affected individuals and 12.5% of unaffected relatives. Of the affected females, there were a total of 43 successful pregnancies and 7 miscarriages giving an incidence of miscarriage of 14%; similar to the overall population miscarriage rate of 0-15%. There were a total of 10 successful pregnancies in the unaffected females and 3 miscarriages (incidence of miscarriage 23%).

This study of 17 families demonstrates a small increase in bleeding manifestations in patients affected with Arg-16-His mutation as compared to unaffected relatives but no increase in thrombotic or pregnancy complications.

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### **In vivo recovery of ristocetin co-factor and in patients with von Willebrand disease undergoing procedures requiring treatment with Haemate P**

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It is important to ensure that the dose of clotting factor concentrate patients receive is within the therapeutic range to minimise risk of bleeding or thrombosis in patients undergoing invasive procedures.

This study looked at the data in patients with von Willebrand disease (VWD) who underwent procedures requiring Haemate P treatment between 2002-2006. We obtained a minimum dataset of weight, dose of factor concentrate, age, sex, pre and post first dose of concentrate levels for factor VIII and ristocetin cofactor (VWF:RCO) and type of VWD on 30 patients for 33 episodes.

There were 23 episodes with females and 10 with males. Twenty-one had type 1 VWD, 10 type 2 and 2 type 3. Median age was 45 (range 21-84). Mean dose of Haemate P was 37.6 units of factor VIII per kilogram (range 22.5-54.5) equivalent to 90.2 VWF:RCO units.

Data showed a wide range of results with a mean recovery of 1.50 IU/dl per IU/kg of VWF:RCO administered (standard deviation = 0.55, range 0.56 to 2.43). There was no significant difference in recovery between the mean of males (1.48) and females (1.50). There was an inverse correlation (-0.55) between increasing doses per kilogram and recovery. There was some correlation (0.36) between increasing weight and recovery. There was no correlation with age.

Our study showed that recovery cannot be predicted for individuals. There is poor correlation between recovery and age or sex of the patient. There was a fall in recovery with larger doses of concentrate per kilogram and there appears to be greater recovery in larger patients. The mean recovery of 1.50 IU/dl per IU/kg of VWF:RCO administered is the same as stated in the Haemate P summary of product characteristics.

The study shows the importance of dosing based on VWF:RCO levels in pre and post operative patients.

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### Managing peri-delivery anticoagulation in women on therapeutic dose low-molecular weight heparin: a role for unfractionated heparin

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There is no evidence based approach for the optimal management of peri-delivery anticoagulation in women receiving therapeutic dose low molecular weight heparin (LMWH) during pregnancy, although maintenance of anticoagulation for the maximal period peri-delivery appears appropriate in these women considered to be at high risk of venous or arterial thromboembolism. We developed a simple, flexible regimen based on fixed prophylactic dose unfractionated heparin (UFH) peri-delivery and audited the outcomes. The study population comprised 33 consecutive women who received therapeutic dose LMWH during pregnancy, with the indications: venous thromboembolism (VTE) in index pregnancy/high risk of VTE (26) and cardiac: mitral stenosis/prosthetic valves (7). On the evening prior to planned delivery (induction of labour or elective caesarian section (CS)), fixed prophylactic dose UFH by continuous intravenous infusion (15,000units/24 hours) was commenced, starting 12 hours after the last dose of 12 hourly LMWH. UFH was stopped between 1–6 (typically 4) hours pre-delivery and restarted 2–6 hours post-delivery. LMWH (75% of therapeutic dose) ( $\pm$  warfarin) was reinstituted 24–48 hours post-delivery, and UFH stopped. Nineteen patients were delivered by CS (9 elective), of which 6 received epidural/spinal anaesthesia without complication. In 28/33 patients managed according to the specified regimen, there were no cases of PPH (blood loss >500 ml or >1000 ml within 24 hours of vaginal delivery or CS respectively) and no thrombosis. In the remainder (5/33), there was one case of PPH and one line-associated thrombosis immediately after post-natal emergency mitral valve replacement. In conclusion, the peri-delivery anticoagulation regimen based on fixed prophylactic dose UFH enabled maintenance of anticoagulation except for a matter of hours, and, was simple, flexible and acceptable to staff and patients.

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### Off-label use of rFVIIa and its clinical effectiveness: results from a Scottish national audit

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Recombinant FactorVIIa (rFVIIa; Novoseven) is licensed to treat bleeding in haemophilic patients with inhibitors. Anecdotal reports and randomised studies indicate its potential value in intracerebral haemorrhage (ICH) and massive haemorrhage. During 6 months in 2006, the Scottish Haematology Audit Network audited patterns for off-label use of rFVIIa in 27 acute Hospitals. Results were compared with a previous audit from 2003.

Ninety-two per cent of the hospitals keep a stock of rFVIIa on site, 48% having a written protocol for its release. In 88%, Consultant Haematologist authorisation is required. Off-label use has increased since 2003 (48 vs 38 episodes/year) especially in non-Haemophilia Centre Hospitals. Other than increasing use for ICH, indications have changed little over time. Immediate response rate [RR; defined by either major reduction or cessation of haemorrhage according to subjective clinical assessment], 24-hour survival rate [24SR] and 28-day survival rate [28SR] have not changed – combined data showing 68% RR, 79% 24SR and 52% 28SR. A high coagulopathy score [CS; scoring one point for each of platelet <50, fibrinogen <1.0, and INR or APTTr > 1.5] is associated with non-significant lower RR and 24SR. Combining data from both audits, comparing CS 2–3 vs CS 0–1 by Chi-squared test gives RR of 58% vs 78% and 24SR of 62% vs 84% ( $P < 0.1$  for both). Platelet count <50, alone, is associated with significantly worse RR and 24SR – 45% vs 83% ( $P < 0.01$ ) and 60% vs 85% ( $P < 0.05$ ) respectively.

In conclusion, off-label use of rFVIIa has increased in District General Hospitals, reflecting its wider availability. It has also started to be used for ICH. rFVIIa appears less effective in patients who are more coagulopathic, particularly if platelets are <50 at time of administration. Overall, despite a high subjective clinical RR of 68%, 28-day survival is poor at about 50%.

## Free Communications: Red Cell Disorders and Transfusion

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### Reports to the Serious Hazards of Transfusion (SHOT) scheme of post-transfusion purpura (PTP) and transfusion-associated graft-versus-host disease (TA-GvHD) before and after implementation of universal leucocyte depletion in the UK

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To establish whether universal leucodepletion in the UK has reduced the observed incidence of PTP and TA-GvHD, all reports to SHOT from 1996–2005 meeting these case definitions were reviewed. For TA-GvHD, the case numbers transfused with LD vs non-LD components were compared. Since the LD status of components transfused to PTP cases was unknown, we compared case numbers

before and during implementation of universal LD (1996–1999), with 2000–2005.

From 1996–2005, 6.2 million non-LD and 19.6 million LD red cells and platelets were issued. There were 13 reports of TA-GvHD, all fatal. Ten patients had at least one risk factor: undiagnosed immunodeficiency (2), B cell lymphoproliferative disease (6), steroids (1), transfusion of fresh blood (1) and donor/recipient HLA haplotype share (4). Eleven cases received non-LD red cells and/or platelets and 2 LD ( $P < 0.001$ ). No TA-GvHD cases have been reported to SHOT from 2002–2005.

There were 46 reports of PTP; 45 in parous females, one in a previously transfused male. Two patients died. Alloantibodies to the Human Platelet Antigen-1a (HPA-1a) were found in 34 cases (78%), either alone (29), or in combination with other HPA antibodies (2), heparin-induced antibodies (2), or a glycoprotein Ia/IIa autoantibody (1). Nine cases had antibodies to other HPA specificities, either singly or in combination. From 1996–1999, there were 31 PTP cases (10.3/year), which fell to 15 (2.5/year) from 2000–2005 ( $P < 0.001$ ).

Before universal LD, only 1/31 (3%) PTP cases had received platelets as well as red cells, in contrast to 8/15 (51%) afterwards ( $P < 0.001$ ). Prior to LD, 11/31 cases (32%) had antibodies to HPA specificities other than HPA-1a, compared to 1/15 (6.5%) after LD ( $P = 0.07$ ).

These findings suggest that universal LD has markedly reduced but not eliminated the risk of TA-GvHD, and has both reduced and altered the profile of PTP cases.

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### Selection criteria and consent for granulocyte donation

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The use of Granulocyte Colony Stimulating Factor (G-CSF) has revived interest in granulocyte transfusion as a strategy to protect against bacterial or fungal infection in neutropenic patients.

Local and national guidelines were used as standards to audit donor selection when volunteer donors (relatives and friends) were referred for G-CSF, Dexamethasone and apheresis granulocyte donation. The donation program was coordinated between the Bristol BMT unit and a blood establishment.

All donors must answer a screening questionnaire and undergo a medical assessment by a doctor other than the physician treating the potential recipient. Donors should be ABO, RhD and CMV compatible with the patient (and in SCT to the stem cell donor). All donors must undergo mandatory microbiology screening. National guidelines also suggest that all recipients be screened for HLA antibodies.

Ninety-five potential donors (age range 20 to 68 years) were referred. Ten failed screening criteria because they were either underweight, too old, had high titre anti-A, a raised aspartate transaminase, were HLA incompatible with a refractory recipient or in four cases they were CMV IgG+. Therefore 85 donors donated 169 donations for 38 recipients.

The average dose of granulocytes obtained was  $58.1 \times 10^9$ /pack (range 2–134). Only 3 doses were less than  $10 \times 10^9$ /pack, one dose being less than UK specification of  $5 \times 10^9$ /pack. One donation was ABO incompatible (donor O+ with high titre anti-A, recipient A+). This donation was plasma depleted. All apparently CMV incompatible donations were justified by the clinical scenario (CMV negative recipient ineligible for allogeneic transplantation or CMV –/– graft in whom the risk of mortality from sepsis outweighed the risk of CMV transmission).

Adherence with guidelines was confirmed in over 90% of donations the apparent exceptions were clinically justified. Acceptable doses of granulocytes can be obtained for most recipients following the implementation of a robust program for donor selection.

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### Type I recessive congenital methaemoglobinaemia associated with a triple mutation of NADH-cytochrome b5 reductase

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Recessive congenital methaemoglobinaemia (RCM) is caused by NADH-cytochrome b5 reductase (*cb5r*) deficiency. Two distinct clinical forms have been recognised. In both types I and II cyanosis is present, but patients with type II also exhibit neurological impairment. The enzyme is composed of one FAD and one NADH binding domain linked by a hinge region. It is encoded by the *DIA1* gene and more than 40 mutations have been described, some of which are common to both types of RCM. Mutations associated with type II tend to cause incorrect splicing, disruption of the active site or truncation of the protein. To investigate the contribution of specific mutations to the two clinical phenotypes a heterologous expression system has been developed. We have used this to investigate a child aged 7 years with type I RCM with two different mutations, the novel Pro92His on both alleles and the previously described Glu255del on one allele. To assess the impact of the Pro92His and the Pro92His/Glu255del double mutation on the function of *cb5r* both variants were generated using the expression system and characterised for enzyme activity and thermostability. The Pro92His mutation, located in the FAD binding domain, moderately impaired enzymatic activity of *cb5r* without causing dramatic changes in affinity for NADH. The Glu255del mutation, located in the NADH binding domain, strongly reduced enzymatic activity. Moreover, the thermostability of the Pro92His variant was substantially decreased as indicated by reduction of the T50 by 11°C compared to wild type enzyme. The Pro92His/Glu255del double mutant exhibited substantial loss of enzymatic activity, lowered affinity for NADH and decreased thermostability. These experimental data are consistent with the apparent type I clinical phenotype of the child and suggest that further studies using this model system will help to define the characteristics of the enzyme variants which determine the clinical phenotype.

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### Blockade of intravascular haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with the terminal complement inhibitor eculizumab unmasks low-level haemolysis potentially occurring through C3 opsonisation

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PNH is an acquired haemolytic anaemia characterised by intravascular haemolysis. PNH red blood cells (RBCs) lack two complement

regulatory molecules, CD59 and CD55. Eculizumab, a monoclonal antibody that inhibits terminal complement by binding C5, effectively controls intravascular haemolysis determined by a dramatic reduction in lactate dehydrogenase (LDH). Control of intravascular haemolysis in these patients led to a reduction or cessation of transfusions. During eculizumab treatment, most patients demonstrate residual, low-level haemolysis with slightly elevated LDH levels, low/undetectable haptoglobin levels, and above normal bilirubin levels. This low-level residual haemolysis may be due to C3b-mediated clearance of PNH RBCs. We investigated C3 deposition on RBCs in PNH patients before and on eculizumab. A direct antiglobulin test (DAT) using monoclonal anti-C3d was positive in 29/39 PNH patients on eculizumab. Of these 29 DAT-positive patients, who were all receiving transfusions prior to eculizumab, 25 had DAT testing before therapy and only one was positive. Using flow cytometry with anti-CD59 and anti-C3, most patients on eculizumab demonstrated three RBC populations: (i)CD59 + /C3- (normal RBCs); (ii)CD59-/C3- (PNH RBCs without C3 coating); (iii)CD59-/C3+ (PNH RBCs coated by C3). No CD59 + /C3+ RBCs were observed. Of 21 DAT-positive eculizumab treated patients tested, the median proportion of total RBCs that were C3b positive was 17.6%. 18/29 (62%) DAT-positive eculizumab patients received at least one transfusion during eculizumab therapy compared with 1/10 (10%) for DAT-negative patients ( $P = 0.01$ ). The median haemoglobin value for the 29 DAT-positive eculizumab patients was 9.8 g/dl compared with 11.3 g/dl in the 10 DAT-negative eculizumab patients ( $P = 0.08$ ). It is proposed that resolution of intravascular haemolysis in PNH patients on eculizumab results in deposition of C3b on the surface of PNH RBCs which may explain, at least partly, the residual low-level haemolysis occurring in some patients. This is a previously undescribed mechanism of RBC clearance in PNH, most likely obscured by the rapidity of intravascular haemolysis in the absence of eculizumab therapy.

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### The first *ALAS2* alternate splice variant associated with X-linked sideroblastic anaemia (XLSA)

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A 24 year old Swedish male with no declared family history of anaemia presented with fatigue and was found to have a pyridoxine-refractory microcytic, hypochromic anaemia with iron overload (Hb 8g/dl, MCV 55fl, MCH 16pg, serum ferritin 1000 µl/l) and ringed sideroblasts. Sequence analysis demonstrated *ALAS2* variation hemizygoty at codon 190 in exon 5 (GAT > TAT, c.G620T), absent in 184 control alleles, predicting both the Asp190Tyr substitution on the protein outer surface and the insertion of a new splice donor site. The proband's reticulocytes expressed RNA utilising either the normal exon 5 splice donor site or the alternate site removing 72 bases from the 3' end of exon 5. The deletion was in frame and predicted the loss of 24 amino acids (a.a.190–213) comprising a beta sheet, loop and helix sequence linking the *ALAS2* N-terminal and catalytic domains and including at least one invariant residue. A previously-reported mutation (c.A621T; Asp190Val) was shown to inhibit both interaction of *ALAS2* with succinyl-CoA synthase and its correct mitochondrial processing, but should not result in the splice variant observed with Asp190Tyr. The proband's haematologically-normal mother was confirmed to be a c.G620T heterozygote,

however, expression of the variant allele was not detected in her peripheral blood reticulocytes and markedly-skewed X-chromosome inactivation of the abnormal allele was found. Family study on the maternal side has so far revealed four haematologically normal people (3M,1F) without this mutation, one additional female carrier and, in particular, one distantly-related, iron-loaded hemizygote with microcytic, hypochromic anaemia (Hb 7 g/dl, MCV 64fl, MCH 18pg, ferritin 767 µl/l) providing strong evidence for a causal link with XLSA. Investigation of the respective roles of the two *ALAS2* variants in SA is underway, nevertheless this is the first *ALAS2* variant shown to alter RNA splicing that is associated with, and likely to be the cause of, XLSA.

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### Non-invasive prenatal diagnosis for HbSC disease

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HbSC is a genotype causing sickle cell disease (SCD). Prenatal diagnosis (PND) of fetuses at risk is normally carried out by chorionic villus sampling (CVS) which has a 1% risk of miscarriage. The detection of foetal cell-free (cf) DNA in the circulation of pregnant women opened up the possibility for non-invasive prenatal diagnosis and prognosis of many clinical conditions.

A method was developed based on genotyping of cfDNA using matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). The approach was applied to exclude the foetal inheritance of the paternally derived diseased allele. Absence of the paternally diseased allele would reassure the parents while its presence could mean that the fetus is a carrier for the parental mutation or a compound heterozygote for both parental disease alleles.

Eight carrier women (HbAC or AS) at risk of having a fetus affected with HbSC were blood sampled in the first trimester of pregnancy and cfDNA was extracted from their plasma. They subsequently delivered and the genotypes of the babies were confirmed by DNA analysis.

The paternal diseased allele was not detected in cfDNA of five cases (two of these babies were normal and three heterozygous for the mother's diseased allele). The paternal diseased allele was detected in cfDNA of three cases (two babies were HbSC and 1 heterozygous for the paternal diseased allele). These results suggest that in the 5 cases where the paternal diseased allele was not detected the CVS could have been avoided.

This is the first non-invasive prenatal diagnosis approach for exclusion of fetuses at risk of sickle cell HbSC disease.



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**Nocturnal hypoxaemia is associated with conditional and high risk transcranial doppler velocities in children with sickle cell disease**

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**Background:** Snoring appears to be associated with high internal carotid/middle cerebral artery velocities on transcranial Doppler (TCD) in the general paediatric population but there are few data comparing overnight oximetry in those with normal or abnormal TCD. Snoring and low oxyhaemoglobin saturation (SpO<sub>2</sub>) are common in sickle cell anaemia (SCA) but any association with abnormal TCD has received little attention.

**Methods:** Children with SCA in East London had overnight pulse oximetry and regular TCD scans. 45 had sleep studies as part of an

unselected cohort studied between 1991 and 1993 (only 5 of whom had no history of snoring) and the remainder were undertaken as part of clinical care. Mean and minimum SpO<sub>2</sub> were compared in those with standard risk, conditional (>170 <200 cm/s) or abnormal (>200 cm/s) TCD.

**Results:** One hundred and forty-three children (86 boys; median [range] age 6.8 [1–23] years) had TCD and a sleep study. 121 TCDs were standard risk, 15 were conditional and seven were abnormal. In multivariable binary logistic regression, lower mean and minimum overnight SpO<sub>2</sub> were independently associated with conditional or abnormal TCD (for mean SpO<sub>2</sub>, odds 0.9 (95% confidence intervals [CI] 0.7, 0.9),  $P = 0.001$ ; for minimum SpO<sub>2</sub>, odds 0.97 (95% CI 0.95, 0.99),  $P = 0.0001$ ) but age was not. In the 64 patients in whom haematocrit was available, low values were not commoner in those with abnormal TCD ( $P = 0.4$ ).

**Discussion:** TCD velocity >170 cm/s, an intermediate endpoint which predicts a high risk of stroke in SCA, is commoner in snoring children with SCA and sustained and intermittent overnight oxyhaemoglobin desaturation, probably independently of haematocrit. Early management of sleep disordered breathing might prevent the development of cerebrovascular disease in SCA.